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The Female Specific Protein And Reproduction In The Lobster, Homarus Americanus

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THE FEMALE SPECIFIC PROTEIN AND REPRODUCTION IN THE LOBSTER, *Homarus americanus*

by

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ABSTRACT

Interest in lobster aquaculture has demonstrated the need for information on the factors controlling reproduction and growth, since lobsters continue to molt and grow after reaching sexual maturity. This study examines lobster (Homarus americanus Milne-Edwards) vitellogenesis and oviposition, with particular emphasis on the interaction between molting and reproduction. Data were obtained from biochemical and immunological studies on serum proteins from animals in all stages of molt and reproduction.

Both molt and oviposition were found to occur between June and November, but the timing of the two events was asynchronous: molt, oviposition, can occur in any one season, depending on the size of the lobster within the population, suggesting that each process is independent, although interrelated.

Vitellogenesis occurs largely in the 2-4 month period prior to oviposition, and is characterized by the presence in the serum of a female specific protein (FSP), immunologically identical to a major yolk protein (MOF) extractable from mature oocytes. FSP was detectable in the serum from non-ovigerous, intermolt females with ovaries in stages II-V (which represent the stages when the oocytes are enlarging due to the accumulation of yolk), and was maximal at stage III (12.6 mg/ml hemolymph). In late premolt females, ovaries up to stage III were found, but FSP levels were low (less than 1.0 mg/ml serum), suggesting that premolt events inhibit vitellogenesis. The site of synthesis of the FSP

is unknown but is probably the hepatopancreas.

The mandibular organ (MO), a glandular structure with cells resembling those synthesizing steroids or lipids, was examined to determine whether this structure represents a crustacean analog to the corpus allatum, a gland that modifies synthesis of female specific proteins in insects. Bilateral MO ablation resulted in low total serum protein levels over the long term, but no short or long term effect on FSP levels, and no effect on either the occurrence of oviposition or the duration of the intermolt period. In view of this result, the mandibular organ yields a poor analogy with the insect corpus allatum. It is likely that control of the female specific protein in crustaceans lies in either the eyestalk neurosecretory cells or in the ovary itself.

INTRODUCTION

The American lobster (Homarus americanus) is the most valuable marine species landed in Canada, and the lobster fishing industry is an important part of the economy of the east coast of Canada and the United States. Recent interest in lobster aquaculture has demonstrated the need for thorough understanding of the physiological mechanisms regulating reproductive processes in the lobster in order to set up successful 'farming' techniques.

Crustacean reproduction occurs seasonally, and is integrated with the molt cycle. Most crustaceans, unlike insects, continue to molt after reaching sexual maturity; therefore, molting activities must be considered in any study of reproductive control mechanisms. Control of ovarian maturation and molting must be integrated to allow mating and oviposition to occur at appropriate times during the molt cycle.

Reviews of crustacean physiology (Carlisle and Knowles 1959; Passano 1960; Highnam and Hill 1969; Tombes 1970) have considered the process of molting (ecdysis) and its control in some detail, but have only superficially dealt with reproduction, in spite of the fact that experimental evidence exists linking the neurosecretory eyestalk hormones, molt inhibiting hormone (MIH) and ovarian inhibiting hormone (OIH) with control of ovarian development (Adiyodi and Adiyodi 1970). Removal of the source of MIH and OIH (by eyestalk ablation) in immature animals results only in an accelerated ecdysis, but the same operation in mature females of many species (reviewed by Sochasky 1974) results in precocious vitellogenesis and oviposition with a concomitant extension of the intermolt period. This evidence

strongly suggests that somatic and reproductive growth are antagonistic (Bliss 1966), with the two processes being influenced by the hormones of the eyestalk neurosecretory cells.

Passano (1960) suggested that the control of ecdysis in Crustacea might be analogous to that described for insects. He pointed out that in both crustaceans and insects, neurosecretory hormones either inhibit (crustaceans) or stimulate (insects) epithelial glands in the thorax, which, under the appropriate environmental stimuli, secrete a hormone that induces premolt events. Passano (1960) further postulated that control of reproductive processes in crustaceans might also be analogous to that in insects; that a crustacean corpus allatum analog would be found which mediates the effect of eyestalk hormones on ovarian development in a manner similar to the way in which the corpus allatum controls egg maturation in some insects. The existence of a crustacean corpus allatum analog would support the concept of a common control mechanism for molting and reproduction in arthropods.

LeRoux (1968, 1969, 1974) described a 'mandibular organ' (MO) in the mandibulo-maxillary region of 20 decapod crustacean species, and he tentatively suggested that this organ might be the crustacean analog to the insect corpus allatum. This hypothesis was re-stated later by Adiyodi and Adiyodi (1970) although they offered no experimental evidence to support their assumption. Sochasky et al (1972) stated that the mandibular organ may be involved in control of reproduction, and that this organ is a separate structure from the Y-organ (the molting gland of Echalier 1959).

In insects, oocyte maturation is characterized by increasing levels of a female specific protein in the hemolymph (first described by Telfer 1954) which is immunologically identical to the major yolk protein extractable from mature oocytes. In most insects that have been studied, the corpus allatum either stimulates synthesis of the female specific protein in the fat body, or facilitates its uptake by the oocytes (Engelmann 1970; Pratt and Davey 1972). Female specific proteins have also been demonstrated in several crustacean species (Horn and Kerr 1963, 1969; Adiyodi 1968a,b,c; Kerr 1966, 1969; Barlow and Ridgeway 1969; Wolin, Laufer and Albertini 1973; Croisille et al 1974; Fyffe and O'Connor 1974). Hormonal control of the synthesis and/or uptake of crustacean female specific protein by oocytes has not been demonstrated in detail, but, if the role of the mandibular organ is as a corpus allatum analog in Crustacea, then its effect in modifying the production of female specific protein, and ultimately, vitellogenesis needs experimental verification.

This study examines vitellogenesis and oviposition in the female lobster, Homarus americanus, with particular emphasis on the relationship between molting and reproduction, the effect of the molt cycle on vitellogenesis and oviposition, and the role of the mandibular organ in modifying these processes. Data were obtained from electron microscopic studies of the mandibular organ, and from biochemical and immunological studies of hemolymph proteins from animals in all stages of molt and reproduction, some of which were observed for two years.

MATERIALS AND METHODS

Animal handling and holding conditions

Lobsters (Homarus americanus Milne-Edwards) were obtained from (1) Conley's Lobster Company, St. Andrews, New Brunswick; (2) Maritime Packers Limited, Caribou, Nova Scotia; or (3) Silverstein's Fish Market, London, Ontario. Animals were from two geographical stocks: (1) Northumberland Strait, and (2) southern Nova Scotia. Retailers kept the animals in outdoor pounds or indoor holding tanks for varying times until purchase.

Lobsters used for long-term experiments were kept at the Biological Station (Fisheries Research Board of Canada), St. Andrews, New Brunswick in fiberglass tanks measuring 0.9 x 0.9 x 0.3 meters. Drainage tiles were provided as shelter for the lobsters. The sexes were randomly mixed, and no more than twelve animals were placed in a single tank. If an animal was a recent molt and too soft to band, it was assigned a number and kept in an individual tank until the shell hardened enough to allow handling and banding. A constant flow of salt water either heated, or at ambient temperature was supplied. Every four days, the animals were fed a mulch of shrimp, herring, sea urchin, and cod. Excess food was periodically siphoned from the tanks. Tanks were checked daily for dead or freshly molted animals. To prevent cannabalism, freshly molted males were immediately removed from the community tanks and placed in tanks divided into individual compartments. Freshly molted females were allowed to remain in the tanks for twenty-four hours

to maximize the chance that mating would occur. After this period, the females were also placed in the compartmentalized tanks. In both cases, the animals were returned to the community tanks as soon as they had hardened enough to be handled and re-numbered.

Some lobsters used for short-term experiments were kept in London, Ontario in fiberglass tanks, approximately 1.0 x 2.0 x 1.0 meters supplied with re-circulated Instant Ocean solution at 13-15° C. Under these conditions, animals were fed beef liver chunks or pieces of frozen shrimp and lobster. Maximum holding time was one month.

Measurement and identification of animals

Calipers were used to measure the carapace length (the distance from the rear margin of the eye socket to the posterior margin of the carapace, parallel to the midline) and the abdominal width (the distance across the widest part of the second abdominal segment) on each animal. Other distinguishing features such as missing limbs and unusual shell markings were recorded as an aid in identifying individuals. Colored rubber bands were placed around both chelipeds at the base of the dactyls, thus immobilizing them. Identifying numbers were printed on these bands with a permanent ink marker pen or numbers were etched into the dorsal surface of the carapace using a Dremel tool equipped with a dental bit.

Sexual characteristics

Female lobsters can be readily distinguished from males by examination of the first abdominal pleopods. In the male, these pleopods are large, chitinous structures, modified for insertion

into the female seminal receptacle at mating. Females have a much reduced set of first pleopods, smaller even than those on the remainder of the abdominal segments. The morphology of the remaining abdominal segments of males and immature females is indistinguishable; however, mature females show a marked increase in relative abdominal width when compared to males or immature females as well as a marked increase in the length of the pleopod setae (ovigerous setae used in securing the egg mass to the maternal abdomen).

Sexual maturity

Templeman (1935, 1944) found that the ratio of abdominal width to total length of the lobster increased rapidly with the approach of sexual maturity, thus providing a method for determining the approximate maturity of female lobsters from a wide variety of geographical areas where the actual size at maturity might vary considerably. Since modern measurements are based on carapace length rather than total length, Templeman's method was modified to permit abdominal width/carapace length ratios (AW/CL) to be used to estimate maturity of females used in this study.

The ovarian factor

In addition to the morphological criteria, the mass of the ovary can be used as a distinguishing characteristic of female sexual maturity; however, absolute ovarian weights cannot be used to compare ovarian stages from lobsters of different sizes. Ovarian weight must be expressed in relation to some measure of the lobster's size or weight. Total body weight is not a reliable comparison since

lobsters, especially those held in community tanks, often autotomize limbs. Accordingly, an ovarian factor (O_f) was derived, based on the weight of the ovary and the cube of the carapace length (the carapace length is a more reliable index of size). The formula used (Aiken, personal communication) is as follows:

$$\text{OVARIAN FACTOR } (O_f) = \frac{\text{ovary weight (mg)}}{(\text{carapace length in cm})^3} \times 10$$

Numbers obtained from this formula ranged from 9-400, and represent the full range of the developmental stages of the ovary. The O_f values are summarized and compared to descriptions of the ovaries in Table III. It should be emphasized that ovary factors were always used in conjunction with the morphological criteria in assigning an ovarian stage, since the values of the O_f for each of the ovarian stages varied widely.

Molt staging

Stages of proecdysis were determined by examination of fresh, severed tips of the pleopods with a compound microscope according to the criteria established by Aiken (1973, Appendix I). With this method, animals can be accurately staged in their progression through proecdysis. If a lobster was between ecdysis and stage C_4, the criteria in Table I were used.

Hemolymph sampling and preparation of serum

Lobster hemolymph samples were obtained with a 3-ml Plastipak disposable syringe (Becton and Dickson) equipped with a 20-gauge needle. The needle was inserted just under the ventral membrane of the first or second abdominal segment, lateral and parallel to the

Table I

Criteria for staging lobsters between stages A and C_4 inclusive*

Molt stage		Description
A	Postmolt	up to 24 hours postmolt, carapace soft
B	Postmolt	tips of chelipeds hard; carapace slightly flexible; i.e. 'paper shell'
C_1	Intermolt	carapace flexible at all points
C_2	Intermolt	carapace hard in dorsal cephalic region, flexible elsewhere
C_3	Intermolt	carapace hard on posterior dorso-lateral surface
C_4	Intermolt	carapace completely hard, pleopod examination consistent with that of Aiken (1973)

* After Aiken (unpublished)

ventral nerve cord. Hemolymph from the ventral abdominal sinus was slowly withdrawn into the syringe, and immediately expressed gently down the side of a 15-ml Corex centrifuge tube. The tube was swirled gently until a visible clot formed and the sample cleared. The tubes were then covered with Parafilm (American Can Company) and placed at 4° C for 8-10 hours to allow complete clotting. The clot was loosened with an applicator stick and the tubes were centrifuged for 10 minutes at 10,000 x g at 10° C in a Sorvall SS-3 centrifuge. The resulting clear serum was decanted with a Pasteur pipet into individual, numbered BEEM capsules (with lid, Fullam Scientific Company). Serum samples were frozen at -20°C for up to eighteen months without any noticeable change in clarity or volume.

Serum protein determinations

Total serum protein levels were determined using the method of Lowry et al (1951), or the Biuret method of Henry et al (1957). Standard curves of different concentrations of bovine serum albumin (Sigma Biochemicals, Fraction V) were constructed for each test; for the Lowry test, the curve included values from 10 to 500 µg per ml, while for the Biuret test, the curve was made for concentrations from 1.0 to 8.0 mg per ml. All optical density determinations were performed on a Bausch and Lomb Spectronic 70 spectrophotometer, with glass cuvettes. For the Lowry method, readings were taken at 750 or 500 nm, while for the Biuret test, readings were taken at 550 nm.

Disc gel electrophoresis

Lobster serum and ovarian proteins were separated electrophoretically on 7% polyacrylamide gels using the method of Davis (1964) and Ornstein (1964) (see Appendix II for stock solutions and buffers) except that no loading gel was used because of problems with obtaining polymerization in the presence of lobster serum. Each run was performed at 5 to 10° C. The volume of sample applied to each gel was adjusted so that between 100 and 180 µg protein were present. Volumes loaded were usually less than five µl.

After removal from the electrophoretic apparatus, gels were removed from their tubes by gently rimming them with a blunted syringe needle. The gels were cut at the dye mark and immediately fixed and stained in a solution of 0.5% Amido black (BDH Chemicals) in 7% aqueous acetic acid, for 1-8 hours. Gels were destained electrophoretically by placing them in 7mm (ID) tubes (12 cm long) plugged at one end with 1 cm of 7% polymerized acrylamide gel. Seven per cent aqueous acetic acid was used to fill the tubes, as well as to fill the upper and lower reservoirs. The apparatus used for destaining was identical to that used for the original electrophoresis except that carbon electrodes were used. Currents of 8 mA per tube were applied for about two hours at room temperature. Destained gels were stored in fresh 7% aqueous acetic acid in stoppered vials. Some gels were stained with Sudan black B (for lipid), or with the periodic-acid-Schiff reagent (for carbohydrates) according to the methods described in Sargent (1969, page 14).

Stained gels were scanned on a Photovolt Densicord densitometer Gels were placed in glass tubes filled with 7% aqueous acetic acid and were scanned without filters on mode 5 or mode D-2. Both these settings are quasi-logarithmic, giving pen deflections on the recording proportional to stain absorbance. The tracing obtained was expanded 1:5 in comparison to the original gel dimension. Areas under the curves obtained were estimated by using the Integrator apparatus attached to the Densicord. (This device records marks on the tracing proportional to pen deflection). Stained gels were photographed on 35-mm Panatomic-X film (Kodak) using fluorescent back lighting.

Isolation and purification of the major ovarian protein fraction

Fragments of ovarian tissue were homogenized in a Teflon homogenizer in 10 volumes of ice-cold 0.5M NaCl containing 5 mM EDTA. The homogenates were centrifuged at 20,000 x g for 20 minutes, and the supernatant was used for both electrophoresis and subsequent purification. The major ovarian protein fraction (MOF) was isolated from the homogenates of ovaries with an O_2 of 250 (Table III) using the ammonium sulphate precipitation technique described in detail by Wallace et al (1967) except that centrifugation was reduced to 20,000 x g for 30 minutes at 0-5° C. The bulk of the MOF precipitated at 67% saturation of ammonium sulphate (0° C). Purity of the resulting preparations was checked using 7% acrylamide gel electrophoresis as described previously for serum samples.

Sodium-dodecyl-sulphate (SDS) gel electrophoresis

Samples of purified major ovarian fraction (MOF) were separated on SDS polyacrylamide gels (5% acrylamide, 0.2% SDS) according to the method of Weber and Osborn (1969). The sample (10 mg/ml protein) was diluted with 9 volumes incubation buffer (0.01M sodium phosphate buffer (pH7.0), 1% SDS, 1% beta-mercaptoethanol) and incubated at 37° C for 2 hours. Five to fifty µl of the incubated sample (5-50 µg protein) were mixed with 3 µl of 0.005% aqueous Bromphenol blue and applied to the gels. Runs were at room temperature with an initial current of 2 mA per tube for one hour. The current was then increased to 8 mA for another 4 hours. Gels were stained with fresh 0.25% Coomassie brilliant blue R250 (Canalco Inc., Rockville, Maryland) in 50% methanol (containing 10% glacial acetic acid) for 36 hours at room temperature, and laterally destained electrophoretically for 2 hours. Gels were scanned and photographed as previously described for standard gels.

Injection of antigen (MOF) and bleeding of rabbits

The final 67% ammonium sulphate precipitate was resuspended in, and thoroughly dialysed against 0.5M NaCl at 5-10° C. The dialysate was combined 1:1 with Freund's complete adjuvant (Kallestad Laboratories Inc.) in a small beaker and throughly mixed by drawing the solution up into a syringe barrel several times until a drop of the mixture did not spread on a water surface. The adjuvant-protein mixture was prepared immediately before injection. Care was taken not to overcool the emulsion since separation would result.

The antigen-adjuvant mixture was injected into each of four male rabbits three times over a period of six weeks. The first injection was subcutaneous in the hind foot pads (0.5 ml in each pad). The second and third injections were intramuscular into the buttock (1.0 ml). In each case, a tuberculin syringe equipped with a short 20 gauge needle was used. Concentrations of antigen were adjusted so that each rabbit received 5 mg total antigen per injection.

Four weeks after the last injection, two of the rabbits were bled via the peripheral ear vein. The rabbits were placed in a restraining box (similar to the one shown in Campbell et al (1970)). The ear was shaved with a safety razor, coated with a thin layer of petroleum jelly, and warmed with a lamp until the ear vein was visibly dilated. A small slit was made in the vein with a clean, single-edged razor blade and drops of blood were collected in 15-ml Corex centrifuge tubes. Up to 40 ml of blood were obtained per bleeding. The tubes were covered with Parafilm, kept at room temperature for an hour to allow clotting to occur, refrigerated overnight, and centrifuged at 1,500 x g in a Sorvall SS-3 centrifuge at 10° C for 10 minutes. Aliquots of the clear serum were collected with a Pasteur pipet placed in stoppered 5-ml vials, and frozen at -20° C.

Rabbit sera were tested for the presence of antibody to lobster MOF (anti-MOF) with the ring precipitin test described in Campbell et al (1970). Equal volumes of the sera and 10 mg/ml MOF solutions were used and a precipitate was observed at the interface of the two solutions. If the sera were positive with the ring test, the various

bleedings were pooled for further tests.

Immunodiffusion analysis

Serum samples from male and female lobsters, 20,000 x g supernatants derived from 0.5M NaCl homogenates of ovaries, and purified preparations of the major ovarian fraction (MOF) were tested for the presence of antigens precipitable by anti-MOF by diffusion in agar gels on microscope slides following the technique of MacGregor (personal communication). Clean microscope slides were coated with hot 0.1% agar in distilled water (purified, Difco Laboratories) and air dried. Two strips of double thickness vinyl tape were placed on the slides about 2.5 cm apart. Hot 1.0% agar in 0.05M barbital-HCl buffer (pH 7.2) was poured into the space between the tape strips. A square plastic template made from 3 mm thick plexiglass (6 wells drilled in a hexagonal array and one well in the center) was lowered on to the hot agar so as to be supported on the tape strips. The tape supports allowed approximately a 1.0mm layer of agar to remain under the template. The slides were allowed to cool, and the agar was removed from the bottom of the wells with a needle and slight suction with a Pasteur pipet attached to a vacuum hose. Serum (approximately 5 µl) was loaded into the peripheral wells and 5 µl antiserum (anti-MOF) were loaded into the center well. With the template still in place, the slides were placed in closed Petri dishes lined with wet filter paper, and left undisturbed for 24 to 48 hours at either room temperature or at 15° C.

After the incubation period, the templates and the tape strips were removed. The non-precipitated proteins were washed out by immersing the slides in distilled water or 0.9% NaCl at room temperature for 10-12 hours. Slides were stained with 0.5% Amido black in 7% aqueous acetic acid for 1-2 hours, destained by washing in fresh aqueous acetic acid, air dried, and stored in microscope slide boxes.

Slides were photographed on 35-mm Panatomic-X film (Kodak) using fluorescent back lighting.

Immunoelectrophoretic analysis

Samples (sera and purified MOF) to be tested for proteins precipitable by anti-MOF were first run on 7% polyacrylamide gels as previously described. Pairs of gels were then placed parallel to each other, about 4 cm apart, in the bottom of a 10 cm square Petri dish. Warmed 1.0% agar in 0.05M barbital-HCl buffer (pH 7.2) was poured over the gels to cover them. After the agar had solidified, a 1.0 mm x 9.0 cm slit was made in the agar equidistant (2 cm) from and parallel to the acrylamide gels, and the antiserum poured into the resulting trough. After 24-48 hours diffusion at 15-20° C in a humid environment, the acrylamide gels were removed, and the agar plates were placed in 0.9% NaCl for 6-8 hours to wash out the unprecipitated proteins. The agar was stained with 0.5% Amido black in 7% aqueous acetic acid for 1-2 hours and destained by immersion in several changes of fresh 7% acetic acid. The agar gels were air dried and photographed as described for the immunodiffusion plates.

Mandibular organ

1) Ablation. Mandibular organs were bilaterally removed from lobsters with excellent survival. In all cases the MO were ablated postmolt (stage B) or early intermolt (stage C_2-C_3). Animals were restrained on their sides in a home-made wooden clamp which was shaped to the contours of the carapace. A roughly spherical or triangular incision, about 5 mm in diameter, was made in the antero-lateral surface of the carapace just anterior and lateral to the cervical groove. A dental drill was used initially to cut through the calcified shell, followed by a sharp razor blade scalpel. In the case of animals in stage B of postmolt, only a razor blade scalpel was used to ablate the MO. The piece of carapace removed was saved for later repair of the incision. Excess hemolymph was removed by aspiration. The anterior lobe of the hepatopancreas was moved aside with a blunt probe revealing the compact, green to light brown mandibular organ closely apposed to the base of the posterior mandibular adductor muscles. The organ was removed either with suction or with fine forceps. The site of the incision was dried thoroughly with facial tissue and the previously removed piece of carapace was replaced over the incision and permanently secured with a tissue adhesive (Histoacryl-N-Blau, Peder Pederson, Guelph, Ontario). The mandibular organ from the other side was similarly removed either immediately or after a short recovery period. Sham operations were performed exactly as an ablation, except that the mandibular organ was left in place. At the termination of mandibular organ ablation experiments, success of the operation

was carefully checked visually during autopsy and any remaining tissue was weighed to the nearest milligram on a Mettler balance.

2) Microscopy. For light microscopy, mandibular organs from mature males and females (southern Nova Scotia stock) were fixed in Bouin's fluid and paraffin sections were stained with Delafield's hematoxylin and 2% aqueous eosin B.

For electron microscopy, mandibular organs were fixed in one of the following solutions (at 4° C): (a) a mixture of 1% osmium tetroxide and 6.25% glutaraldehyde, buffered to pH 7.4 with 0.063M s-collidine (Trump and Bulger 1966); (b) 10% paraformaldehyde containing 0.122M sucrose, 0.005M $CaCl_2$, buffered to pH 7.4 with 0.07M s-collidine followed by 2% buffered osmium tetroxide (pH 7.4) (Winborn and Seelig 1970); (c) 5% glutaraldehyde buffered with 0.05M cacodylate to pH 7.4 (with 2% sucrose). After a buffered wash, tissue fixed in solution (c) was incubated according to the method of Novikoff and Goldfischer (1969) to demonstrate catalase activity. Control media contained 0.03M 3-amino-1,2,4-triazole to inhibit catalase, or 5×10^{-4} KCN to inhibit mitochondrial cytochrome oxidase. Tissue was postfixed in 2% buffered osmium tetroxide.

All tissue was dehydrated in acetone and embedded in Epon 812 (Luft, 1961). Silver to gold sections were collected on uncoated copper grids and examined unstained, stained for 30 minutes with saturated aqueous uranyl acetate, or doubly stained with uranyl acetate and 0.5% lead citrate (Venable and Coggeshall 1965). Grids were examined at 40 or 60 KV in either a Philips 100C or 201 electron microscope. Micrographs were taken at initial magnifications

of 5-30,000 diameters on either 35-mm Fine grain Positive Film, or 3 1/4 x 4 inch (Kodak) Electron Microscope Film (4489), and subsequently enlarged photographically.

RESULTS

FEMALE REPRODUCTIVE CYCLE

1. Sexual maturity

Measurements of several females carrying eggs ('berried' females) show that although there is a wide variation in carapace lengths, there is a narrow range of AW/CL ratios, all greater than 64% (Table II). All these females were carrying eggs, and therefore definitely mature. Not all sexually mature animals however, must have an AW/CL ratio greater than 64%. Ovigerous females were observed with AW/CL ratios as low as 62%. This ratio is greater though, than that for males. Males examined in this study had carapace lengths from 78 to 89 mm, and their AW/CL ratios were between 52 and 56%. From these measurements, it is clear that if a female has an AW/CL ratio greater than 64%, it is capable of carrying eggs without further molts.

2. Interaction between molting and oviposition

Newly ovigerous females were found to be in either stage C_4 of intermolt (Table I), or in early premolt (stage 1.0, Appendix I), indicating that oviposition occurs in the intermolt period. In order to establish the timing of molting and oviposition, long-term observations were made on lobsters selected from two broad size groups: Group 1, 60-70 mm carapace length; and Group 2, 74-88 mm carapace length. Also, Group 1 were selected for those that had molted in August and September, 1972. Over the following season the peak of ecdysis in Group 1 was in June, with oviposition

Table II

Measurements of carapace lengths and abdominal widths made on ovigerous females from the Northumberland Strait stock.

	Mean carapace length (mm) ±SD*	$\frac{\text{Mean abdominal width}}{\text{Mean carapace length}} \times 100$ ±SD*
Group I	82.07 ± 3.65 (13)	67.6 ± 1.88 (13)
Group II	82.40 ± 3.50 (9)	64.4 ± 2.70 (9)
Group III	73.80 ± 2.30 (6)	69.0 ± 1.05 (6)

*SD represents the standard deviation of the sample.
Numbers in parentheses indicate number of lobsters measured.
Group I-1972; Group II-1973; Group III-1974.

occurring largely in June (Figures 1, 2). Of these lobsters ovipositing in June (12 out of a total of 15 that extruded eggs), all molted by November (Figure 1).

Group 2 lobsters were obtained in early July, 1973. The molting peak in this group was in August (Figure 3), and of the 23 that molted, 17 oviposited by the end of October of the same season (Figure 4). Of the remaining 6 lobsters, 2 survived until the following August, and both successfully oviposited. Those lobsters which both molted and oviposited in August-October 1973, and survived to the following season, either molted or were in late premolt (at least stage D_1 (4.0)) by August, 1974.

From the above observations, the following seasonal patterns emerge: (1) molt only in the spring (Group 1); (2) oviposition in the spring and molt in the late fall (Group 1); (3) molt and oviposition in late summer, followed by molt the next summer (Group 2); and, (4) molt in late summer, followed by oviposition the next summer (Group 2). Deviation from these patterns occasionally occurs when, rather than ovipositing, a female will reabsorb all the yolk from the oocytes. These females are readily discernable since the hemolymph becomes dark green due to the high concentration of yolk protein circulating in the hemolymph. Reabsorbing females were also observed to be in intermolt or in early premolt.

3. The ovary

The lobster ovary is an 'H'-shaped organ lying ventral to the heart and dorsal to other thoracic and abdominal organs. Two ovarian lobes extend forward and lie lateral and dorsal to the anterior lobes

of the hepatopancreas. The other two lobes extend posteriorly into the abdomen. Ventral to the heart, the anterior and posterior lobes are joined by a short transverse lobe. Paired oviducts extend ventrally from this point to open through a pore at the base of each third walking leg.

As the oocytes proliferate and undergo vitellogenesis, they enlarge and move peripherally within the ovarian cavity, thus becoming visible through the thin connective tissue capsule surrounding the ovary. From examination of several ovaries *in situ*, five categories of ovarian morphology can be described, based on macroscopic changes in ovarian size and color (Table III). A combination of the morphological description and the relative ovarian weight (O_f value, Table III) allows a set of ovarian stages to be constructed, as summarized in Table III.

4. The female specific protein

Qualitative and quantitative measurements were made on the proteins in the serum of females to further investigate, and possibly measure, the relationship between molting and oviposition in lobsters.

Hemolymph samples were taken in the summer (July-August) from females derived from the Northumberland Strait stock. The animals were in intermolt, and samples were obtained from females with ovaries in each of the stages described in Table III. Serum proteins were separated on 7% acrylamide gels and densitometric scans of representative gels are shown in Figure 5.* Sera from females

* Some material in both male and female serum samples remained at the surface of the acrylamide gels. There appeared to be approximately the same amount of this material present in each case.

Table III

Ovary stages and descriptions for female Homarus americanus.

Ovarian stage*	Physiological state	Description**	Range of ovarian factors (O_f) observed***
I	immature	white, creamy	9-120
II	post-oviposition, 'resting'	yellow, with 1.0 mm dark green spots scattered throughout; sometimes flaccid	80-166
III	vitellogenesis	light green, no oocytes visible	127-178
IV	vitellogenesis	dark green, 0.5-1.0 mm oocytes visible	200-320
V	mature, ovulation	dark green, 1.5 mm oocytes clearly seen; oocytes free in ovarian cavity	320-400

* Based on similar classification schemes described by Krouse (1973) for Homarus americanus and Bomirski and Klek (1974) for Rhithropanopeus harrisii and Crangon crangon.

** All observations were made in situ.

***After Aiken (unpublished)

$$O_f = \frac{\text{ovary weight (mg)}}{(\text{carapace length in cm})^3} \times 10$$

with ovaries in stages II to V exhibit 10-11 detectable protein bands (stained with Amido Black) with measured R_f values of 0.03 to 0.92. The R_f values of bands 2,4, and 7-10 were measured from 40 gels to determine the reproducibility of the mobility with the electrophoretic conditions used. The R_f values of each of these bands fell within a small range of variance in gels run at various times, thus confirming the reproducibility of the technique (Table IV). The R_f values of bands 5 and 6 were difficult to determine accurately since the resolution of the bands was poor. In a few cases, these two bands became blurred and appeared as a broad single band.

Although serum from females with ovaries in stages II to IV exhibited a distinct protein band with an R_f value of 0.107 (Figure 5), no protein band with this R_f value was detectable in the serum of females with ovaries in stage I or in the serum of any male. Thus, the protein band which has an R_f value of 0.107 is a female specific protein (FSP).

5. Ovarian proteins

a) Crude homogenates. Supernatants from ovarian homogenates (prepared from ovaries with an $O_f > 250$, Table III) were separated on 7% acrylamide gels under the same conditions as for separation of serum proteins. A single major band, with an R_f value of 0.106 and several minor, faster moving bands, were visible (Figure 6a).

b) Properties of the purified fraction. Using the technique of Wallace et al (1967), proteins were precipitated from the 20,000 x g

Table IV

Measured R_f values for some lobster proteins

Bands	Origin	N	Observed Mean R_f value ± SD	Mean R_f value for *Uca pugilator* proteins*
Female specific protein	Female serum	40	0.107 ± 0.012	0.15
"Complex" protein*	Male and female serum	40	0.204 ± 0.071	0.21
Hemocyanin (4 to 5 bands)*	Male and female serum	40	0.809 ± 0.039 to 0.918 ± 0.031	0.736 ± 0.884
Purified major ovarian fraction (MOF)	Homogenate of mature ovary	5	0.106 ± 0.007	-

*Identification after Fielder *et al* (1971).
N=number of gels sampled
All samples were run on 7% acrylamide gels at pH 8.8-9.0 at 5-10°C.

supernatants derived from 0.5M NaCl homogenates of stage III ovaries (O_f>250, Table III). The final precipitate was resolubilized in and thoroughly dialyzed against 0.5M NaCl. Samples of this major ovarian fraction (MOF) containing up to 50 µg total protein were separated on 7% acrylamide gels using the same conditions as for serum samples. A single band with an R_f value of 0.106 was detectable. This band corresponded to the major band resolvable from crude ovarian homogenates (Figure 6a) as well as to the female specific protein (FSP) present in the serum of females with ovaries in stages II-V (Figure 5, Table IV).

The major protein band derived from the MOF stained with Sudan black and with the periodic acid-Schiff reagent, indicating that both lipids and carbohydrates are associated with the major band derived from the ovarian fraction.

When the MOF was preincubated with sodium dodecyl sulphate (SDS) and β-mercaptoethanol, and subsequently separated electrophoretically on 5% acrylamide gels in the presence of SDS, 3 major bands and at least 3 minor bands were observed (Figure 6c).

6. Similarity of FSP and MOF

The similarity of R_f values for the female specific protein (FSP) and the major ovarian fraction (MOF), and the fact that the FSP was only seen in females whose ovaries were enlarging due to the accumulation of yolk suggested a similarity of these two proteins. This similarity was further tested by preparing antibodies to the MOF (anti-MOF). Double immunodiffusion tests in agar gels (Ouchterlony 1958) of the anti-MOF revealed a single precipitin arc

when challenged with the original MOF fraction, or with serum from females whose ovaries were in stages II-V (Figure 7). These arcs were continuous, indicating complete identity of these proteins; however, similar tests with crude ovarian homogenate supernatants showed a second arc (Figure 7). Presence of a second precipitin arc when anti-MOF was tested against the crude ovarian homogenate supernatant indicates that the anti-MOF contained antibodies to more than one protein. Since one arc was continuous with the FSP arc, and the second arc was much closer to the antigen well and was not continuous with the first (Figure 7), the second arc probably represents a minor contaminant of the MOF preparation to which antibodies were made. Male serum did not precipitate the anti-MOF under these conditions.

Anti-MOF was also tested by diffusion in agar against electrophoretically separated samples of purified MOF and female serum which contained FSP. After 40 hours diffusion at 15-17°C, arcs were observed opposite the positions of the FSP and MOF bands respectively (Figure 8). No other arcs were observed under these conditions.

Since the female specific protein (FSP) and a protein in the major ovarian fraction had immunological and electrophoretic similarity, the amount of FSP present in serum samples was quantified by comparing the absorbance of stained bands of FSP in a densitometer with the absorbance values obtained for the band (R_f 0.106) obtained from known amounts of MOF similarly stained and scanned. A curve of absorbance versus known amounts of purified MOF applied to gels is

shown in Figure 9. This curve was used as a standard for subsequent quantification of FSP concentrations in serum samples. It is likely that the MOF was not a single protein (as revealed by the second arc in immunodiffusion tests with a crude ovarian preparation), but since it ran as a single band in acrylamide gels (as did the FSP) the curves were used as a relative standard to make comparative estimates of serum FSP levels in different animals, or in the same animal at different stages. Although no more than 30 µg of MOF could be accurately measured by this method, virtually all the serum samples measured had FSP quantities less than this.

7. Correlation between FSP concentration, molt stage, and ovarian stage.

The quantity of FSP present in serum samples from females with different ovarian and molt stages was estimated using the standard curve established for known quantities of MOF. Once the quantity of FSP was established, the relative concentration (mg/ml) of FSP in these serum samples was calculated. All females were sampled in July-August and were non-ovigerous.

Females in molt stages C_1 to D_0 (pleopod stage 2.0, Appendix I) showed variable concentrations of FSP (Figure 10) with a maximum FSP concentration occurring at ovarian stage III (O_f approximately 175, Table III). The highest FSP value obtained was 12.6 mg/ml serum, which represented 34.5% of the total serum proteins. Females in late premolt (D_1, 3.0) to postmolt (stage B) showed low concentrations of FSP, although ovarian stages up to stage IV (O_f 208, Table III) were observed (Figure 11). No mature (stage V)

ovaries were observed in any of the late premolt lobsters.

8. Seasonal pattern of FSP concentration

Observations were made on female lobsters through a complete year to check the levels of FSP present during normal seasonal fluctuations in ambient temperature. Although FSP concentrations were variable, they can be correlated with seasonal reproductive activity as follows: (1) Molt in August of one year followed by oviposition in August of the second year (Figure 12). In this case, FSP was not detectable until May but steadily increased after this point until oviposition in late August. Water temperature had increased to 7°C in May and was about 15°C by August. The lobster was in molt stage C_4 in September, typical for an ovigerous female. (2) Molt in July followed by oviposition in September of the same year. Detectable levels of FSP were present in the following winter (February) and again in June to August. Water temperatures were at the minimum in February (0°C) and increased from 7° to 15°C from May to August. The ovary was partially developed by September (stage III, O_f 162), indicating that vitellogenesis must have taken place prior to this point. (3) Molt in July followed by oviposition in September of the same year, similar to case 2. In this case, no FSP was detectable in the serum through the following winter and summer (Figure 14), although the water temperature was maximal (15°C) by August. By September, the ovary was observed to be in stage II (O_f 91), indicating that no vitellogenesis had taken place since oviposition the previous year. (In cases 2 and 3 the lobsters molted in August-September in the year after

oviposition, and neither had detectable FSP levels in the month prior to molt (Figures 13,14), an observation which is consistent with the earlier one that FSP levels are low in late premolt).

(4) Molt in July, followed by reabsorption rather than oviposition in November of the same year. FSP shows a characteristic rise and fall that is indicative of the onset of oviposition, but instead, reabsorption occurs, resulting in a marked increase in the concentration of FSP (Figure 15). These high levels persist through most of the winter, when the water temperatures are low, and then disappear just prior to molt in the following September. It is important to note that the timing of molt in this case is no different from that for lobsters which oviposited rather than reabsorbing yolk protein (ie Figures 13,14).

THE MANDIBULAR ORGAN

1. Structure

Lobster mandibular organs are attached to the posterior face of the mandible near the bases of the prominent mandibular adductor muscles (Sochasky et al 1972). They are large (greater than 2 gm in large males), multilobate, pale to dark green structures which have a compact appearance in situ but a flattened, foliaceous structure when freshly dissected. In mature males, the mandibular organ is larger and more resistant to mechanical damage than it is in mature females. A branch of the ventral thoracic artery enters the parenchyma at the ventral surface and its ramifications provide a rich blood supply (Figure 16) to the gland.

The cell population of the mandibular organ varies according to location in the organ (Figure 17). Along the zone of attachment of the organ to the posterior mandibular adductor muscle, the cells are elongate (longitudinal axis about 10 μm) and aligned in rows or cords (Figure 17a). They have a prominent nucleus and lightly staining cytoplasm. Mitotic figures are common and have been observed at all molt stages and in both sexes. Toward the central region of the mandibular organ the cells gradually enlarge (Figure 17b). Nuclei measure up to 10 μm and the amount of acidophilic, granular cytoplasm increases, resulting in spherical and polygonal cells up to 30 μm in diameter. Usually part of each cell surface abuts on a blood sinus, and hemocytes are common in the spaces between the cells (Figures 16,17). No ducts are present in this organ.

At its distal edge, the cells show extensive vacuolization (Figure 17c). The cells boundaries are indistinct and some nuclei are pyknotic.

Although the mandibular organ cells vary in size and degree of vacuolization, their complement of cytoplasmic organelles is the same in both sexes. The fine structure of a typical mandibular organ cell is shown in Figure 18. The granular appearance of the cytoplasm of these cells under the light microscope is due to the presence of numerous mitochondria, microbodies, and less frequent autophagic vacuoles. In the perinuclear region vesicular Golgi complexes and lipid droplets are common and the latter sometimes occur in clusters (Figure 19).

The agranular endoplasmic reticulum of these cells has two morphologically distinct forms: tubular (TER) and cisternal (CER) (Figures 18,21,22). The tubular form is the most common, occurring randomly throughout the cells (Figures 18,21). Occasionally, short segments have ribosomes attached to them. The agranular reticulum appears to proliferate in both sexes during premolt, especially in late premolt (cf Figure 21), resulting in large areas filled with agranular reticulum to the exclusion of other organelles.

The cisternal reticulum is frequently associated with mitochondria or microbodies, and it is rare to find a microbody that does not have at least one closely apposed segment of CER. In some cases, the CER forms extensive layers around a microbody (Figure 22). These 'whorls' are often large and probably correspond

to the round pale areas of cytoplasm with acidophilic centers which are readily seen with the light microscope (W, Figure 17b). These pale areas occur at all molt stages, but are more common in stages D_1 to D_4 of premolt in both sexes.

In addition to the well developed endoplasmic reticulum, the lobster mandibular organ cell often has a convoluted plasma membrane (Figure 20). Its invaginations extend deep into the cell, giving rise to tortuous extracellular channels which appear to be continuous with larger extracellular spaces. The plasma membrane convolutions occur at the surface of cells facing blood sinuses as well as those directly apposed to other cells. Each cell is covered by an external lamina which does not follow the invaginations of the plasma membrane (EL, Figure 20), but does separate the mandibular organ cells from adjacent blood sinuses.

When mandibular organ cells are incubated with diaminobenzidine in the presence of hydrogen peroxide at pH 9.0 and then postfixed with osmium tetroxide, a dense reaction product is found over the microbodies in the cytoplasm (Figure 23), indicating the presence of catalase (Novikoff and Goldfischer 1968, 1969; Locke 1971; Beard 1972). Microbodies have a pale, finely granular matrix, and microbody-like structures, with a more condensed matrix, are often seen (Figure 24). Similar structures contain myelin figures and other debris embedded in their matrix (Figure 24); they do not react with diaminobenzidine and may be intermediate between microbodies and autophagic vacuoles (cf Figure 18).

2. Effects of ablation

Since the FSP in the lobster appears to be a good index of vitellogenesis, the levels of this component of the serum serve as a measurable index of the effects of the mandibular organ on vitellogenesis. Mandibular organs of females were bilaterally ablated and the short and long term effects on serum proteins were studied.

a) Short term effects. Mandibular organs were bilaterally ablated from mature females at stages C_2 to C_3 during midsummer (first week of August). Five females were sham operated, and five females served as unoperated controls. During MO removal, or sham operation, it was possible to observe the ovaries, and in all cases, they appeared to be between stages II and III (cf Table III) indicating that they were probably partially developed at the time of the operation. Animals were maintained at ambient water temperature, which declined from 14 to 7°C during the experimental period. Hemolymph samples (3 ml) were taken from all 15 animals every two weeks and analysed for total protein, and FSP levels.

All lobsters subsequently oviposited (2 reabsorbed) within three months. Concentrations of FSP were similar in the three groups; a gradual increase until just prior to oviposition, and then a rapid decline after oviposition (Figure 25). Total protein levels remained relatively constant during the experimental period and were not different among the three groups (Figure 26).

b) Long term effects. Mandibular organs were bilaterally ablated (December-January) from 25 females (carapace length from 58.0 to 75.0 mm) at stages C_2 to C_3; 13 females were sham operated and 15 females served as unoperated controls. Through the first winter and summer, the lobsters were maintained at ambient temperature (3-15°C). Observations were made on the groups in the first summer and the molting and reproductive events observed are summarized in Table V. The pattern in the three groups is similar; a large percentage molted to maturity (64-74%) while the remainder molted and oviposited (or reabsorbed). MO removal did not appreciably alter the intermolt time (Table VI).

Hemolymph samples were taken monthly from September to June of the second season from animals which had molted to maturity (those lobsters which molted only, Table VI). After December, the water temperature was raised to 10°C and maintained at that level until May, when ambient temperature reached 10°C. At this point, water temperature was allowed to increase at the ambient rate (from 11 to 14°C) until August, when the experiment was terminated. Levels of FSP in the serum were similar in the three groups and showed a steady increase (Figure 27). MO ablation did not have an appreciable effect on the levels of FSP; however, the level of total protein in the serum declined sharply in the MO ablated group (Figure 28). In spite of this sharp decline, one ablated lobster oviposited in April. MO ablation was found to be complete in this lobster.

Table V

Pattern of molting and reproduction in mandibular organ ablated, sham operated, and normal female lobsters.

	Total number	Molt only	Molt-oviposit	Oviposit-molt	Reabsorb-molt
ABLATED	23	17(74)	1(4)	2(9)	3(13)
SHAM	10	7(70)	1(10)	1(10)	1(10)
NORMAL	14	9(65)	2(14)	2(14)	1(7)

All observations were made between May and December, 4-10 months after ablation. Number in () are percentage of total number of lobsters.

Table VI

Mean intermolt time in days to first molt in mandibular organ ablated, sham operated, and normal female lobsters ± SE of the mean

	Total number	Molt only	Molt-oviposit	Oviposit-molt*
ABLATED	23	317.4 ± 3.4(17)	304 (1)	377.8 ± 2.9(5)
SHAM	10	323.6 ± 2.9(7)	292 (1)	336.5 ± 3.6(2)
NORMAL	14	314.7 ± 2.8(9)	297 ± 1.9(2)	392.3 ± 2.7(3)

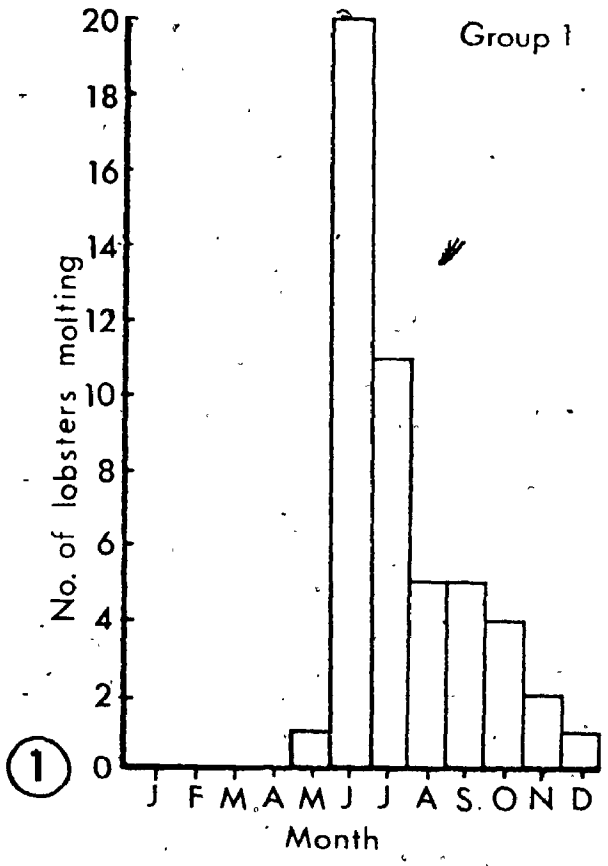
*includes reabsorbing lobsters

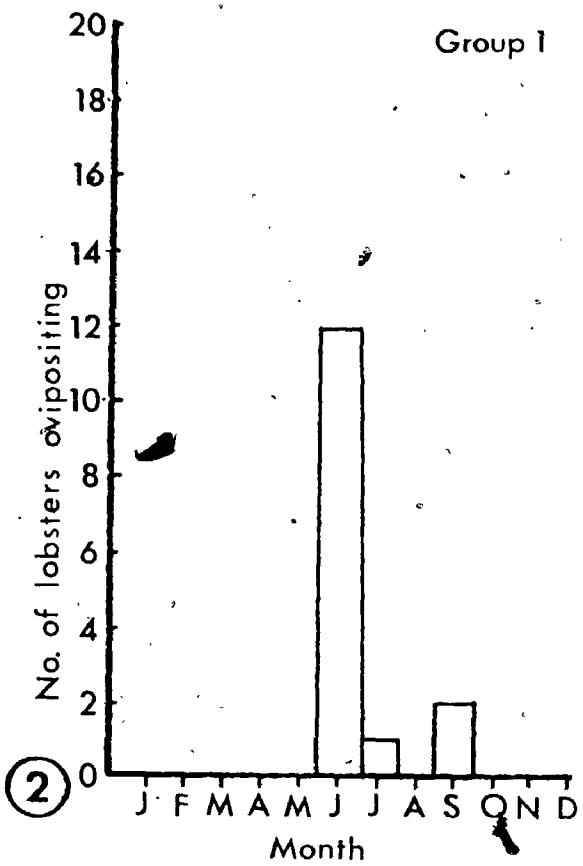
Numbers in () are numbers of lobsters

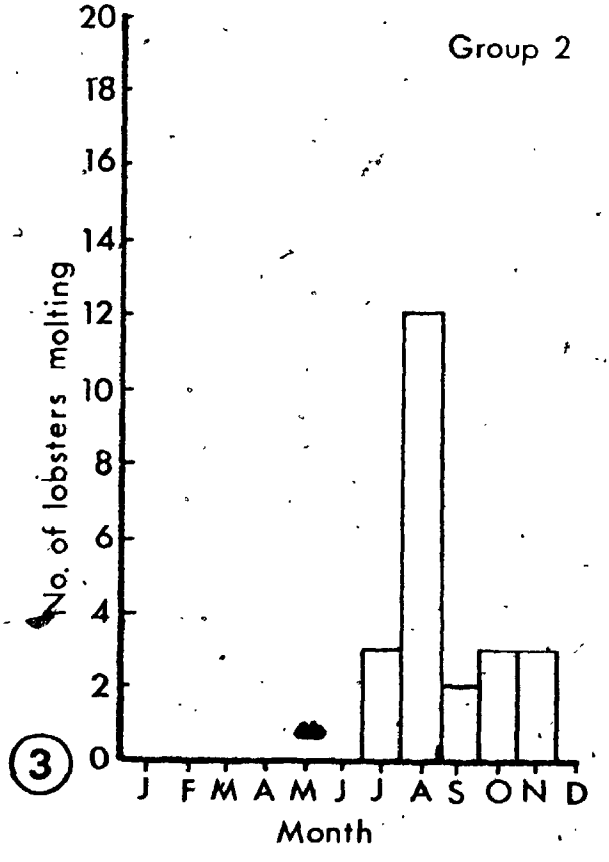
Figures 1, 2, 3, 4: Histograms of molting and oviposition frequencies observed in female lobsters held in community tanks at ambient temperature (3-15°C).

Figures 1 and 2: Group 1 females, 60-70 mm carapace length.

Figures 3 and 4: Group 2 females, 74-88 mm carapace length.


Group 1
No. of lobsters molting
20
18
16
14
12
10
8
6
4
2
0
J F M A M J J A S O N D
Month
1


Group 1
No. of lobsters ovipositing
20
18
16
14
12
10
8
6
4
2
0
J F M A M J J A S O N D
Month
2


Group 2
No. of lobsters molting
20
18
16
14
12
10
8
6
4
2
0
J F M A M J J A S O N D
Month
3

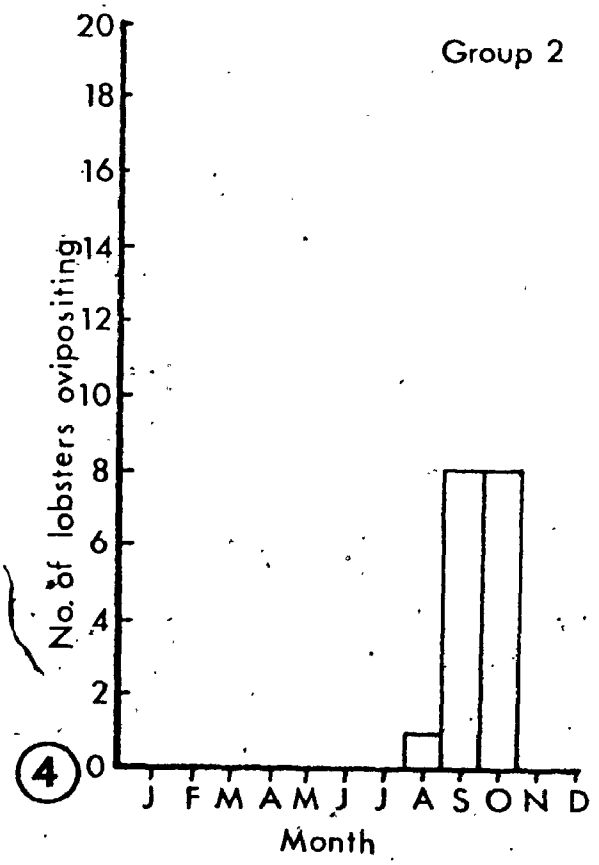
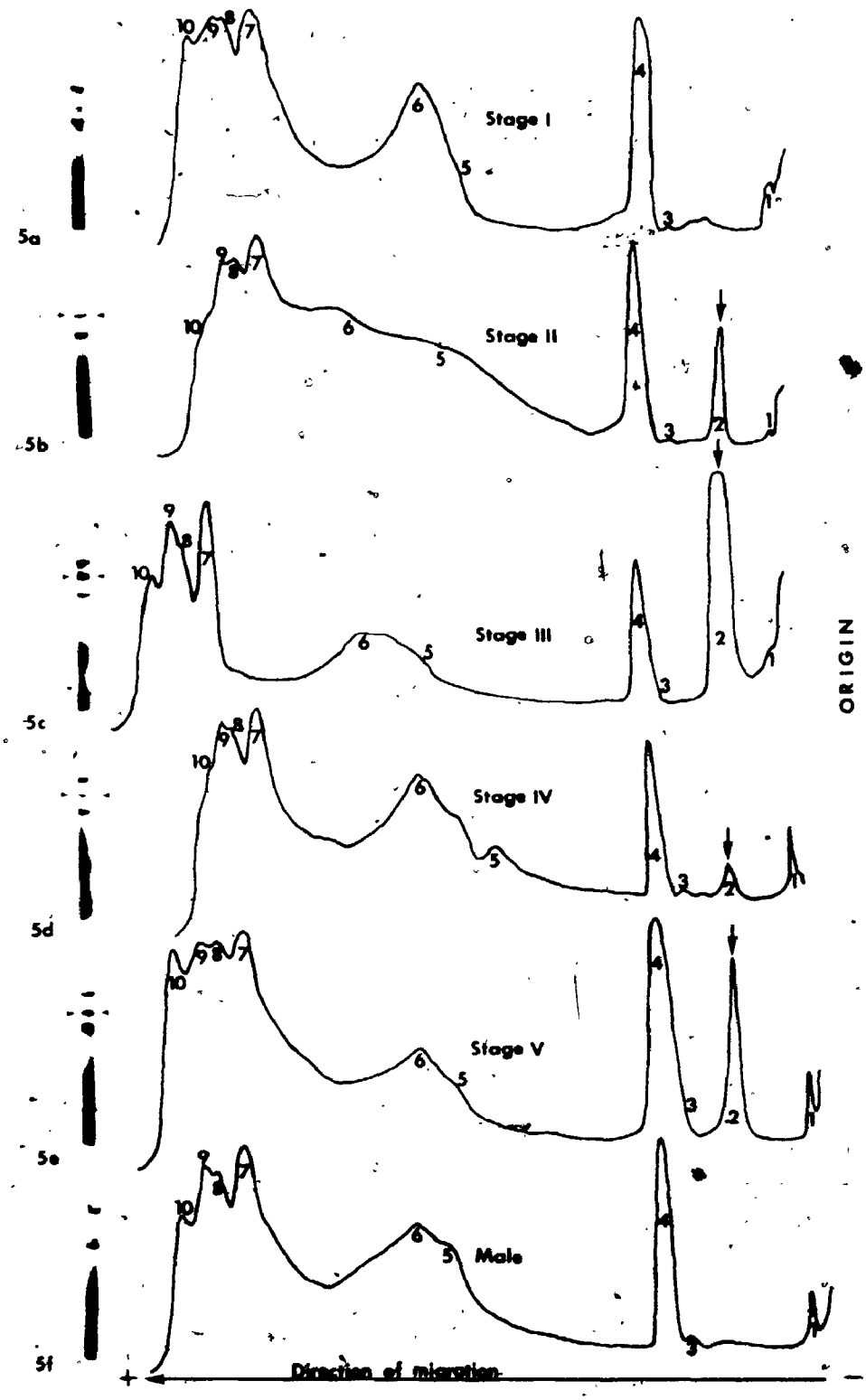

Group 2
No. of lobsters ovipositing
20
18
16
14
12
10
8
6
4
2
0
J F M A M J J A S O N D
Month
4

Figure 5: Representative photographs and tracings of female (a-e) and male (f) serum proteins present after separation on 7% acrylamide gels at 5-10°C (pH 8.8-9.0). Females exhibited ovarian stages from I-V as indicated. Arrows denote the location of the female specific protein (if present) at R_f 0.107. Since varying amounts of protein (100-180 µg) in different volumes (2-5 µl) were separated, only the presence or absence of any band, and not any quantitative relationships, are illustrated.


Stage I
Stage II
Stage III
Stage IV
Stage V
Male
5a
5b
5c
5d
5e
5f
ORIGIN
Direction of migration
+
−

⑤

Figure 6: Densitometric tracings and photographs of proteins present in the 20,000 x g supernatant of 0.5M-NaCl ovarian homogenates (50 µg protein) (a), and preparations of purified major ovarian fraction (MOF) (50 µg protein) (b), after separation on 7% acrylamide gels at 5-10°C (pH 8.8-9.0). The major bands in both cases (arrows) have an R_f value of 0.106. (c) SDS-gel electrophoresis (5% acrylamide) of purified MOF (10 µg protein) reveals that the MOF has (at least) 3 subunits, as well as at least 3 minor components.

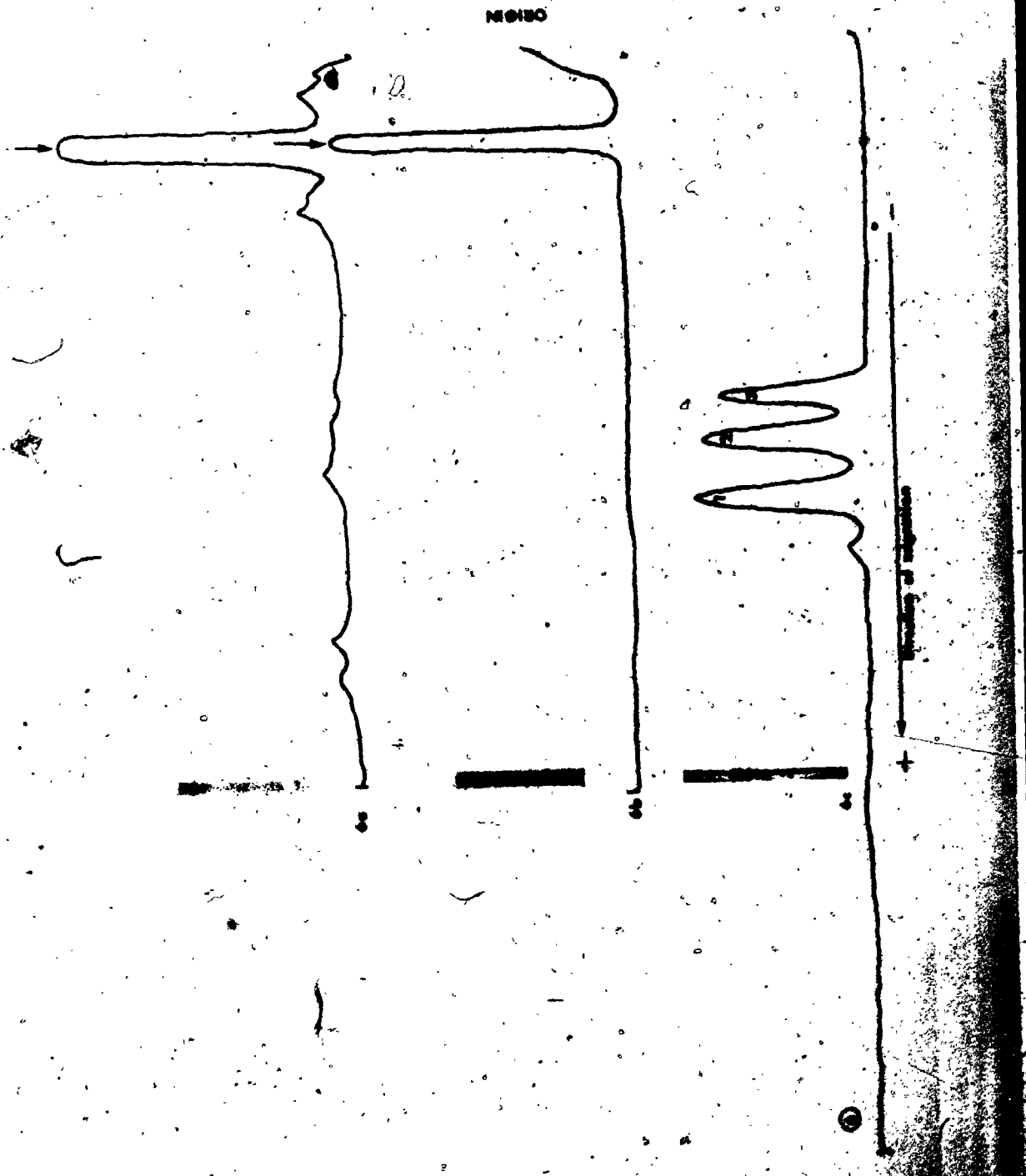
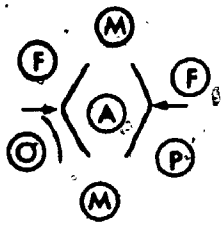


Figure 7: Photograph of a stained, double immunodiffusion slide. A-anti-major ovarian fraction; F-female serum, previously determined to exhibit female specific protein (FSP) on 7% acrylamide gels; M-male serum; O-crude ovarian homogenate supernatant; P-purified major ovarian fraction. Continuity of one of the precipitated arcs (arrows) in each case suggests complete identity of one of the proteins in the F, O, and P wells. The second arc visible at the O well indicates the anti-MOF is specific for another protein in this preparation.
1% agar (pH 7.2), 24 hours diffusion at 15°C.

Figure 8: Diffusion in agar of anti-MOF with electrophoretically separated samples (on 7% acrylamide gels at pH 8.9) of purified MOF (MOF), female serum (FH, 8a), and male serum (MH, 8b). A single precipitin arc is visible in each case, except with male serum (8b), where no reaction is seen.
1% agar (pH 7.2), 40 hours diffusion at 22°C.



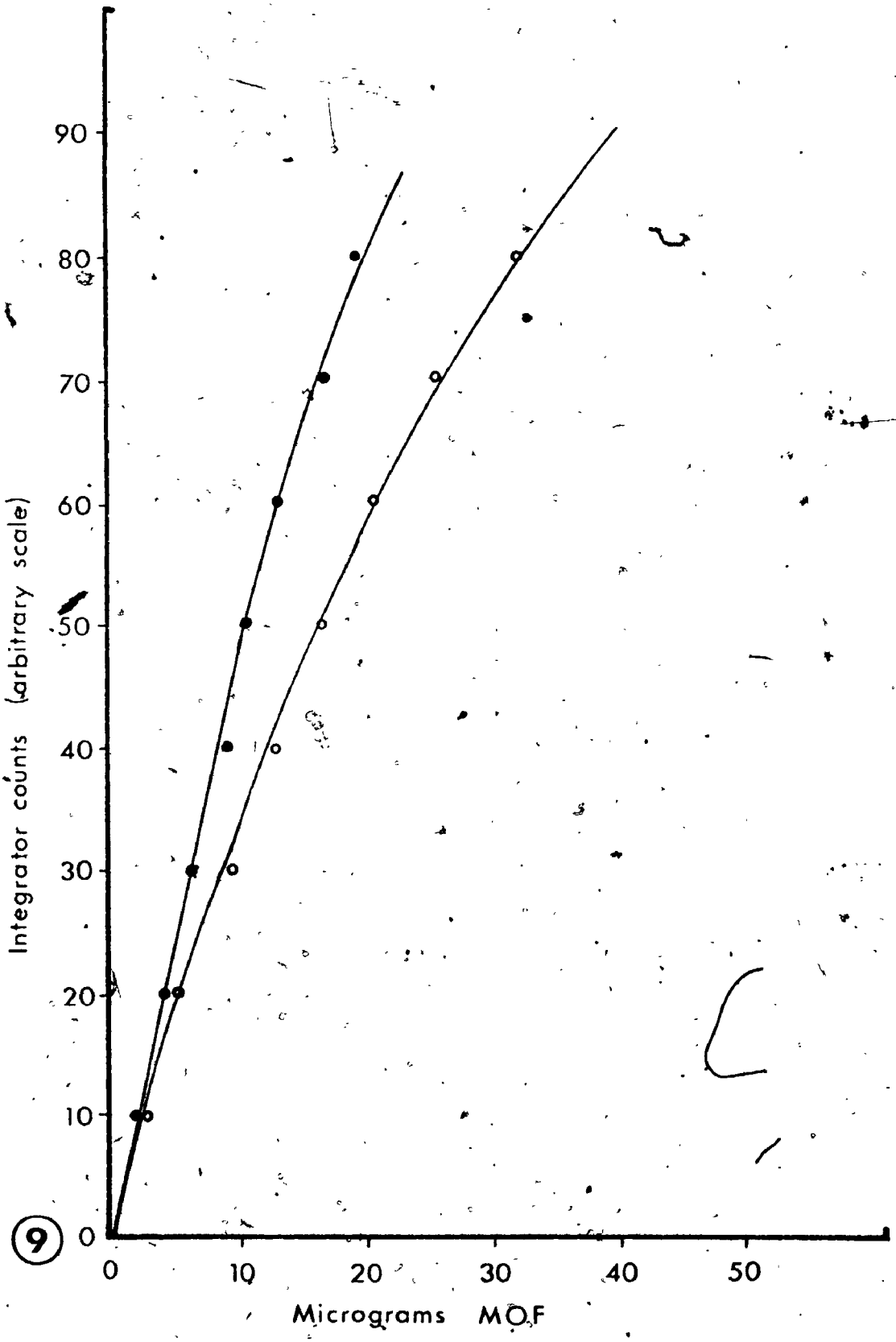
7

8

8a

8b

Figure 9: Standard curves for known amounts of the major ovarian fraction (MOF) versus integrator scores (absorbance) derived from densitometric tracings of samples of purified MOF which has an R_f value of 0.106 when separated on 7% acrylamide gels (pH 8.8-9.0) and stained with Amido black. (●) mode 5, (o) mode D-2.

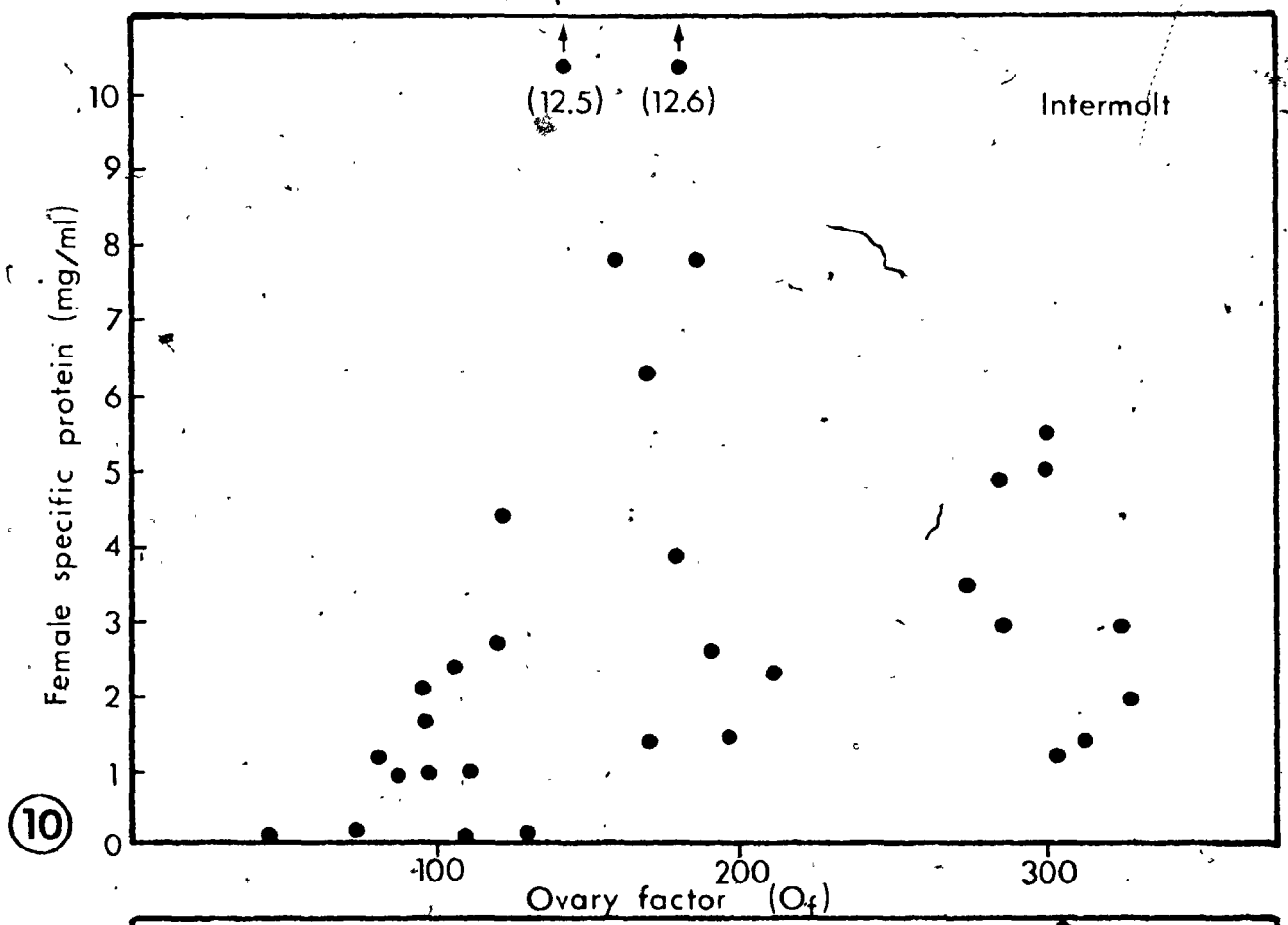

Integrator counts (arbitrary scale)
0
10
20
30
40
50
60
70
80
90
0
10
20
30
40
50
Micrograms M.O.F

(9)

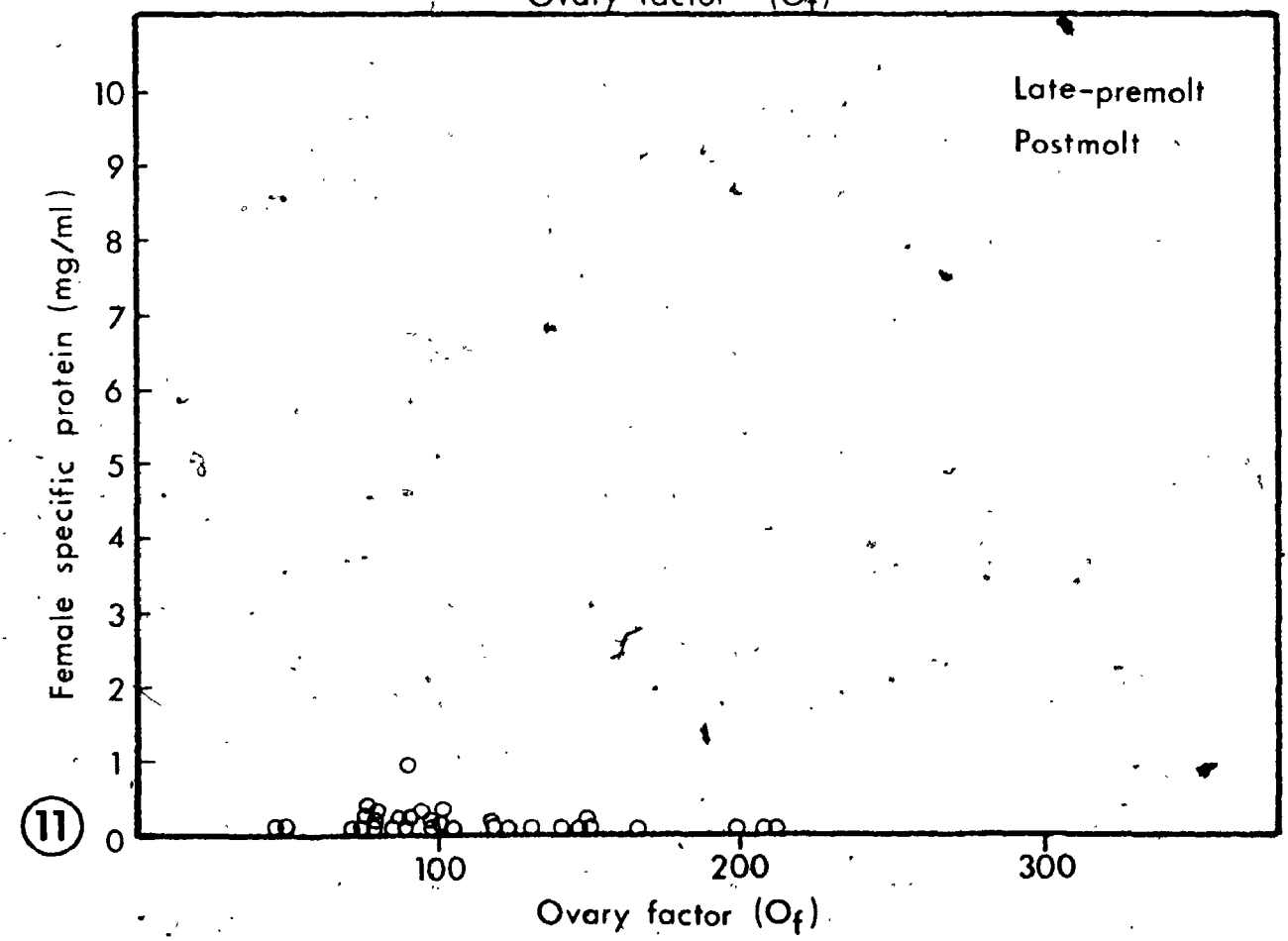
Figures 10 and 11: Correlation between levels of female specific protein and ovarian factor (O_f) in mature, non-ovigerous females. Levels of FSP were determined from quantitation of serum samples separated on 7% acrylamide gels (pH 8.8-9.0). Each point represents a single animal.

Figure 10: Females sampled in July-August in molt stages C_2 to D_0 (2.0).

Figure 11: Females sampled in July-August, in molt stages D_1 (3.0) to stage B of postmolt.


(12.5)
(12.6)
Intermolt
Female specific protein (mg/ml)
0
1
2
3
4
5
6
7
8
9
10
100
200
300
Ovary factor (O_f)

⑩


Late-premolt
Postmolt
Female specific protein (mg/ml)
0
1
2
3
4
5
6
7
8
9
10
100
200
300
Ovary factor (O_f)

⑪

Figures 12, 13, 14, 15: Representative seasonal patterns of female specific protein (FSP) levels in the serum of female lobsters. Each pattern represents monthly samples taken from single animals. Levels of FSP were determined as described for Figures 10 and 11.

M-molt; O-oviposition; O_f-ovarian factor (see Table III); R-reabsorption.

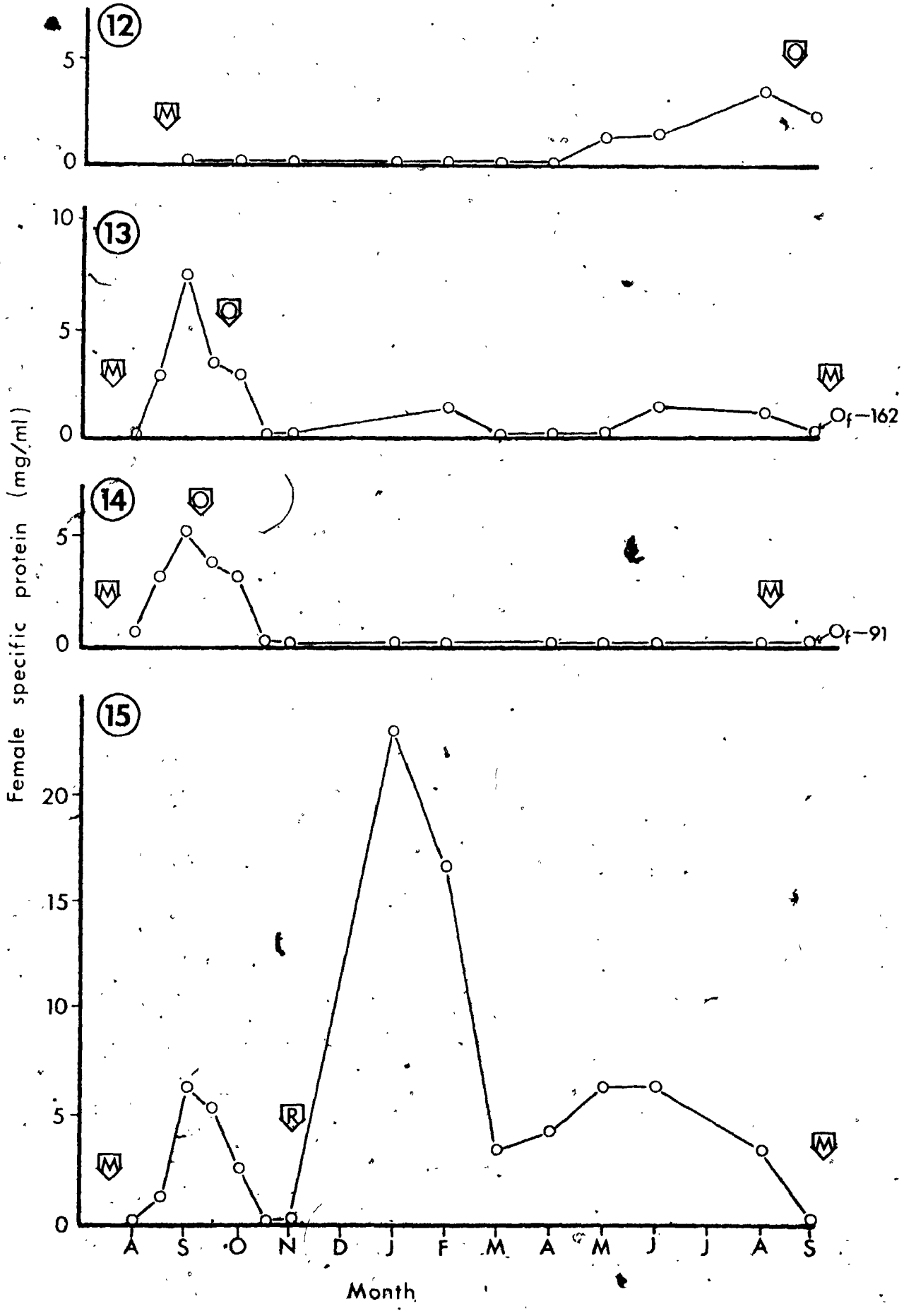

12
13
14
15
Female specific protein (mg/ml)
Month
A S O N D J F M A M J J A S
f—162
f—91

Figure 16: Light micrograph of mandibular organ of male, stage D. Note branching blood vessel (BV) which decreases in diameter (*) as it enters parenchyma. Small branches of this vessel may be continuous with tissue spaces, permitting entry of hemocytes (H) into the parenchyma.

Bouin's fluid Hematoxylin and eosin X 450

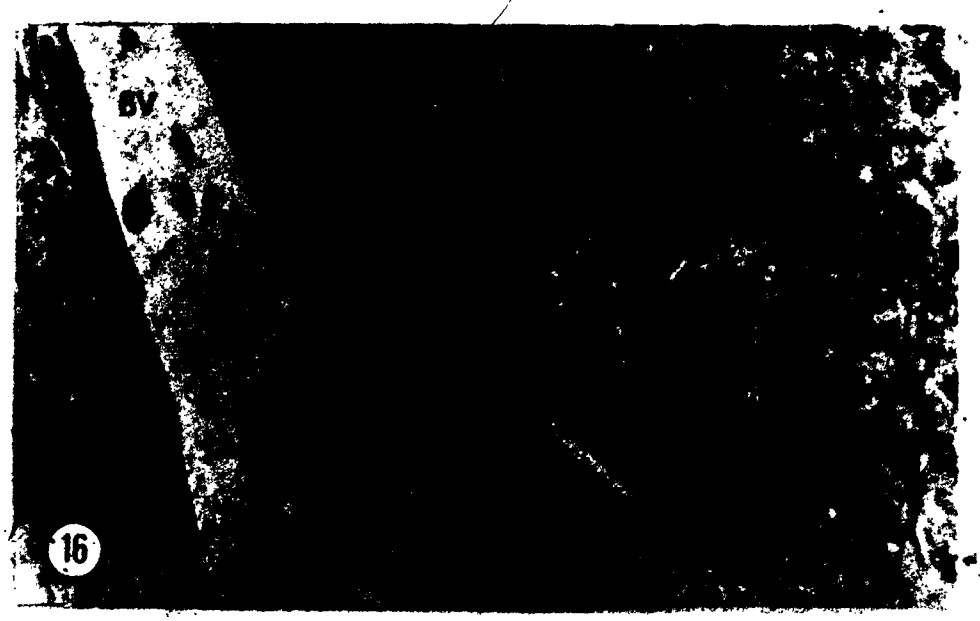

BV
16

Figure 17 a-c: Representative micrographs illustrating regional variability of the cells of the mandibular organ. (a) area close to the attachment of the MO to the posterior mandibular adductor muscle; (b) central region; (c) distal edge. Note mitotic figures (arrows 17a). BV-blood vessel; H-hemocyte; P-pyknotic nucleus; V-vacuole; W-whorl. Female, stage C_4.

Bouin's fluid Hematoxylin and eosin X 625


17a
17b
BV
17c
P

Figure 18: Fine structure of a typical mandibular organ cell. AV-autophagic vacuole; G-Golgi complex; L-lipid droplet; M-microbody; MT-mitochondrion; N-nucleus; NC-nucleolus; TER-tubular agranular endoplasmic reticulum. Male, stage D_0.

Glutaraldehyde-osmium Unstained X 13,485

Figure 19: Perinuclear lipid droplets (L) typically in a cluster.

M-microbody. Male, stage D_2.

Glutaraldehyde-osmium Unstained X 16,000

Figure 20: The external lamina (EL) on the surface of two apposing parenchymal cells. It does not follow the invaginations of the plasma membranes (arrows).

Female, stage D_0.

Glutaraldehyde-osmium Uranyl acetate X 17,000

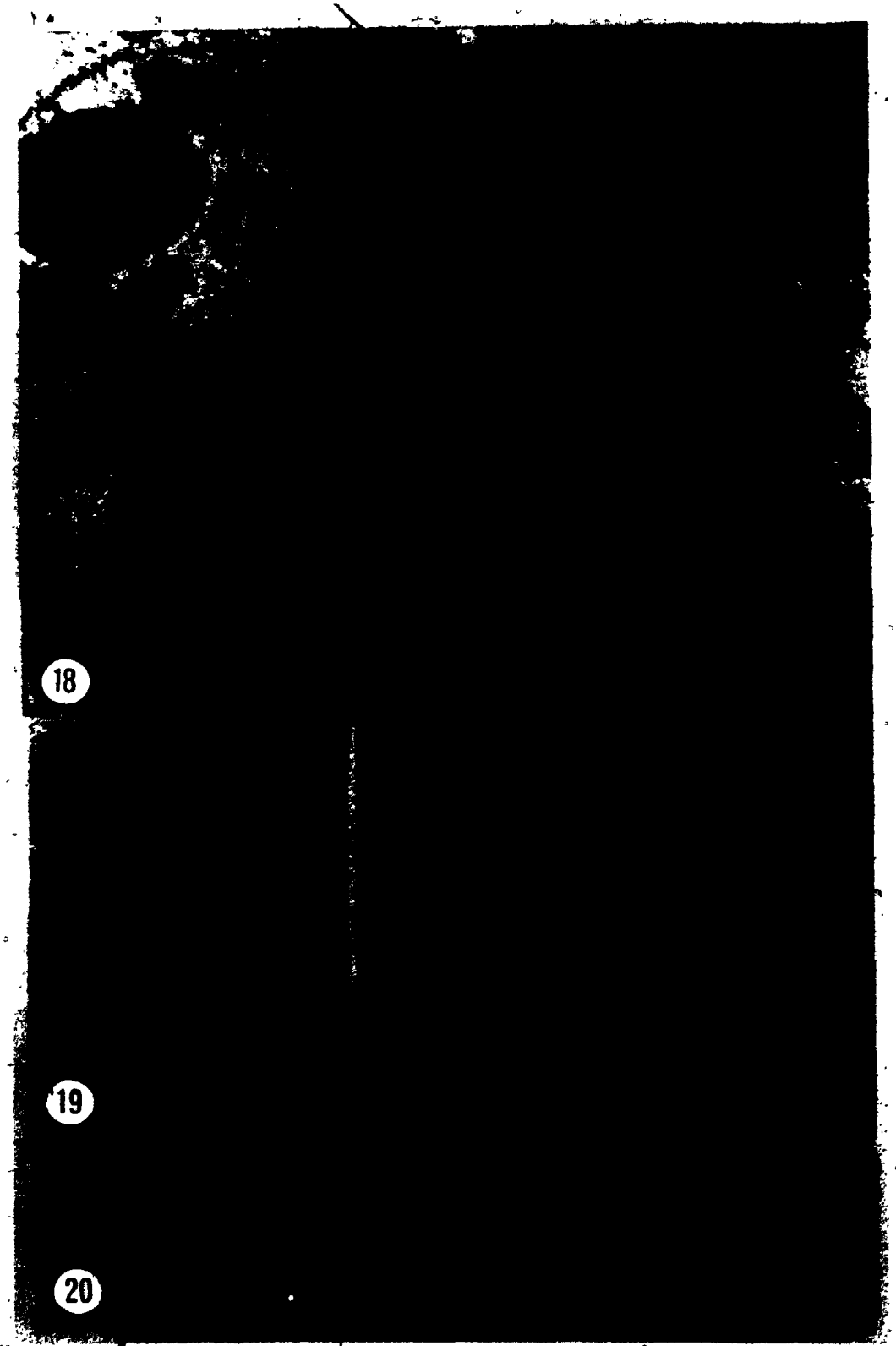

18
19
20

Figure 21: The two forms of agranular endoplasmic reticulum. The tubular ER (TER) is randomly distributed, while the cisternal ER (CER) is in a parallel array. Male, stage D_2.

Glutaraldehyde-osmium Unstained X 19,000

Figure 22: A whorl of cisternal endoplasmic reticulum (CER), completely surrounding a microbody (M). Male, stage D_2.

Glutaraldehyde-osmium Unstained X 15,050

Figure 23: Microbodies (M) are densely stained after incubation in diaminobenzidine plus 5×10^{-5} M KCN, suggestive of catalase activity. The mitochondria (MT) are free of reaction product. Male, stage C_4.

Glutaraldehyde Unstained X 26,500

Figure 24: Structures (arrows) resembling microbodies (M) with a dense matrix and a myelin figure (mf), indicative of autophagic activity. Female, stage D_1.

Glutaraldehyde-osmium Unstained X 13,600

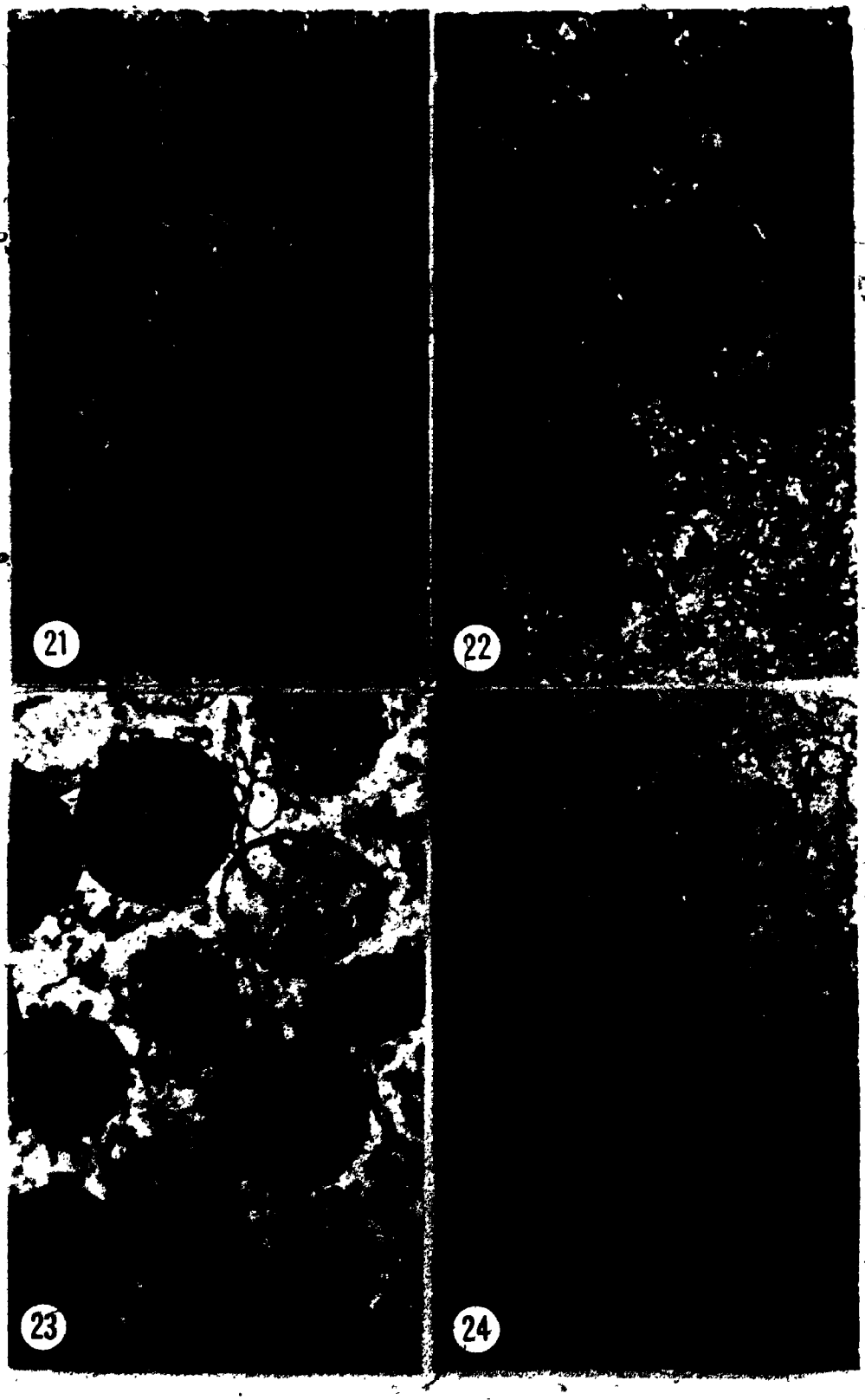
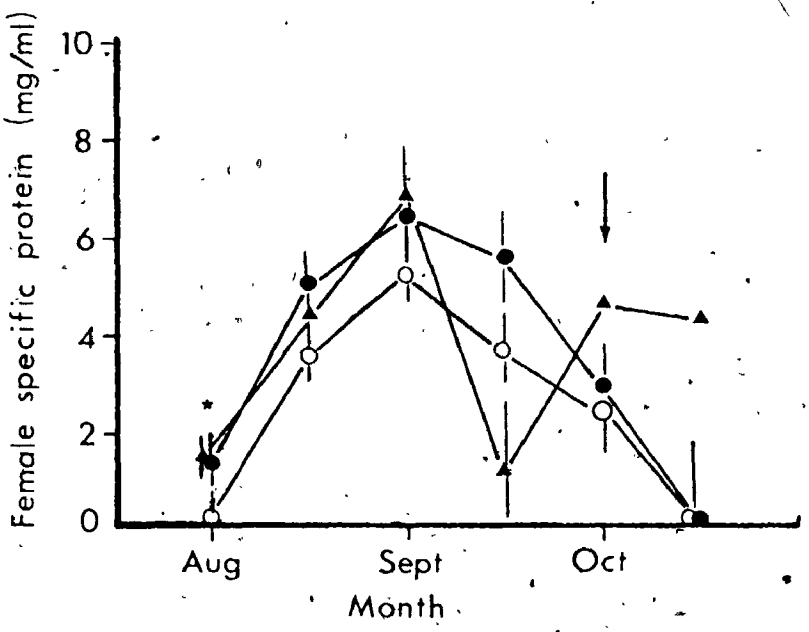
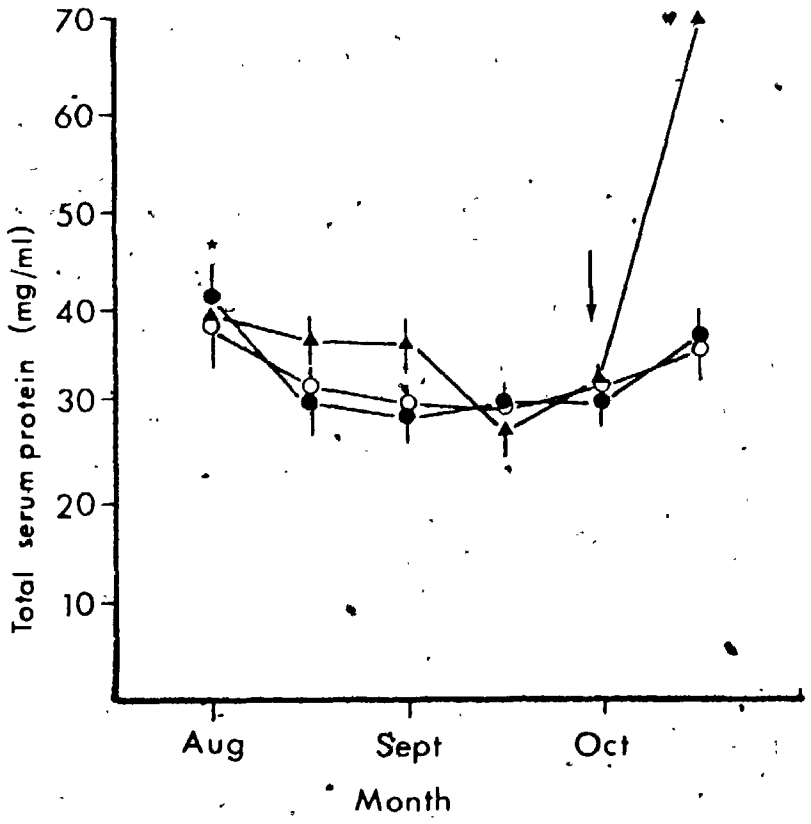

21
22
23
24

Figure 25: Levels of serum FSP in mandibular organ ablated (●), sham operated (o), and normal (▲) mature female lobsters. Ablation was performed in August (*). Each point represents the mean of five animals (except for the last two normal values which represent a single lobster). Arrow indicates the approximate time of oviposition Vertical bars: standard error of the mean.

Figure 26: Total serum protein levels for the same lobsters as in Figure 25. (Last normal point is elevated due to reabsorption in this animal). Arrow indicates the approximate time of oviposition Vertical bars: standard error of the mean


Female specific protein (mg/ml)
10
8
6
4
2
0
Aug
Sept
Oct
Month

25


Total serum protein (mg/ml)
70
60
50
40
30
20
10
Aug
Sept
Oct
Month

26

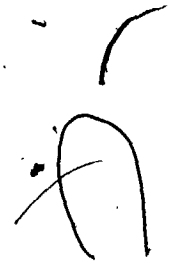
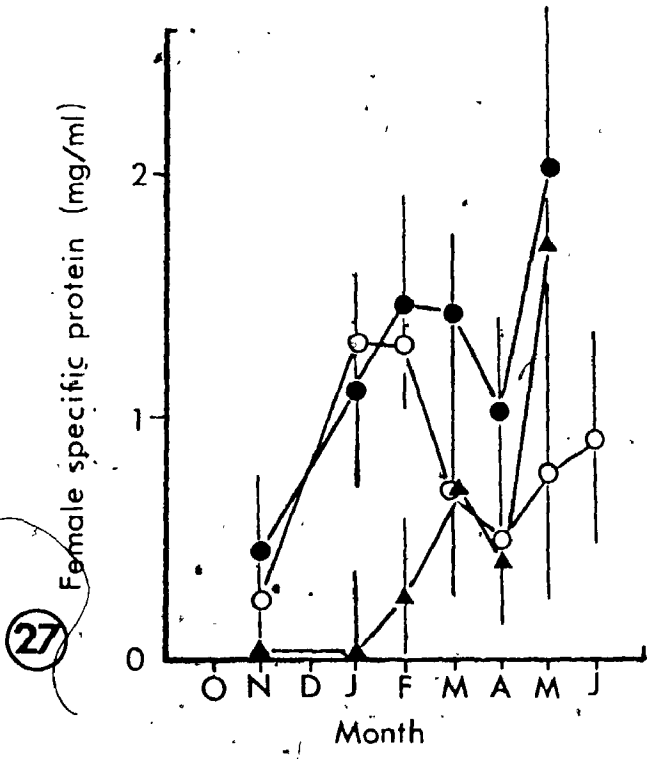
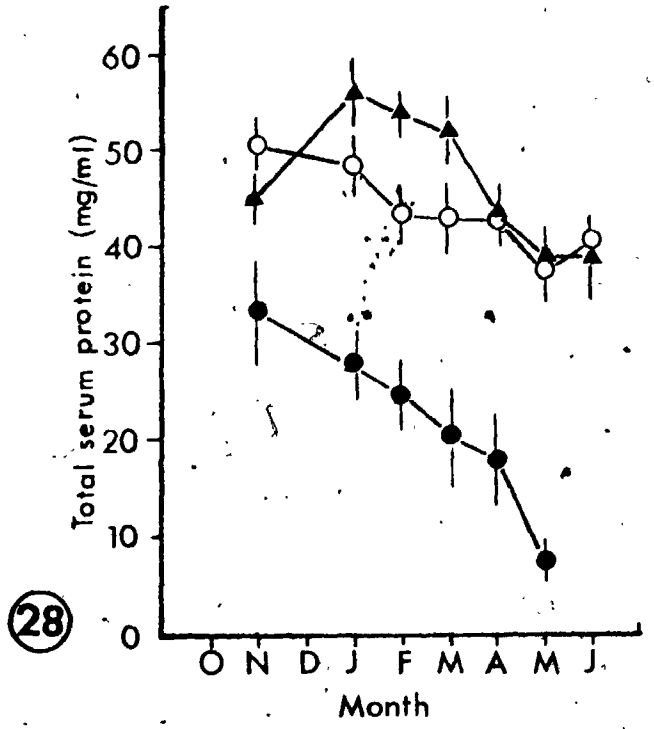


Figure 27: Concentrations of serum FSP in mandibular organ ablated (●), sham operated (o), and normal (▲) female lobsters following the maturity molt. Ablation was performed 10 months prior to the first sample. Each point represents the mean for at least five animals. Vertical bars: standard error of the mean.

Figure 28: Total serum protein concentrations for the same lobsters as in Figure 27. Each point represents the mean for at least five animals. Vertical bars: standard error of the mean.


Female specific protein (mg/ml)
2
1
0
O N D J F M A M J
Month

27


Total serum protein (mg/ml)
60
50
40
30
20
10
0
O N D J F M A M J
Month

28

DISCUSSION

FEMALE SPECIFIC PROTEINS

1. The lobster female specific protein

Lobster female specific protein (FSP), immunologically identical to a major yolk protein extractable from the oocytes (MOF; Figures 7,8) was found in the hemolymph of non-ovigerous, intermolt (or early premolt) females which had ovaries either undergoing vitellogenesis or reabsorption of yolk (Figures 12-15). Varying concentrations of FSP are found in the serum of females in intermolt as the ovary enlarges due to the accumulation of yolk, with the maximum level present at about ovarian stage III (O_f 175; Figure 10). FSP occurs at reduced levels on either side of this maximum, but vitellogenesis, at least as reflected by FSP in the serum, seems to be maximal at ovarian stage III, which is well before full maturation of the oocytes. Adiyodi (1968 b) found a similar pattern of FSP concentration in the crab, Paratelphusa hydrodromous, the maximal level occurring some time before full maturation of the ovary.

In the period from late premolt (stages D_1 to D_3) to postmolt (stage B), non-ovigerous females show low concentrations of serum FSP, even though their ovaries are observed to be in stages I-III, which suggests that premolt events have blocked vitellogenesis and the appearance of FSP (Figure 11). The observation that late premolt lobsters with mature ovaries will reabsorb rather than oviposit, leads to the conclusion that premolt development takes precedence over and will disrupt events leading to normal oviposition.

A similar hierarchy exists in the Thysanuran, Lepismodes inquilinus, an insect species which continues to molt and grow after reaching sexual maturity. In this species, the molting cycle overrides reproductive activity, and artifically induced molting will cause reabsroption of yolk from the oocytes (Rohdendorf and Watson 1969). In this case, and perhaps in the lobster, premolt events and final vitellogenesis and oviposition are incompatible.

2. Seasonal change in lobster FSP concentration

Periodic measurements of the FSP in the serum of lobsters taken over two seasons have shown that vitellogenesis does not normally occur in the winter but during the 2-4 month period prior to oviposition (Figures 12,13,14). In animals where yolk proteins have been reabsorbed, the levels of FSP are much higher (>20 mg/ml serum; Figure 15) than the levels observed during vitellogenesis (5-10 mg/ml serum; Figures 12-14). Concentrations of FSP in the serum of lobsters molting and ovipositing in the same season rise more rapidly and reach a slightly higher level than in those which only oviposit (Figures 12-14). This difference suggests that production of FSP may be more rapid in the former case. Reabsorption of yolk at the beginning of the winter months results in elevated FSP levels for a considerable period of time (6-7 months; Figure 15).

A possible source of error in the quantitation of FSP exists in this study since it seems from the immunodiffusion results (Figure 7) and the SDS gel electrophoresis (Figure 6c), that the MOF fraction, used to construct the standard curve from which

the concentrations of FSP were calculated, probably represents a major protein component plus minor contaminants. These contaminants may have affected the curve sufficiently to result in the actual concentrations of FSP estimated from this curve to be inaccurate. Another source of error results from the fact that some stainable material remained at the surface of the acrylamide gels. This material may have interfered with the migration of FSP or other proteins in the serum. Comparisons of serum FSP concentrations in different animals which have been estimated from this curve, however, probably reflect the actual FSP concentrations.

Densitometric analyses of protein concentrations can be questioned on the grounds that the absorbance of stained protein bands is non-linear at high concentrations; however, since FSP concentrations were calculated from the usable portion of the MOF curve (<30 µg protein, Figure 9) the values obtained permit comparison of relative amounts of FSP.

3. General female specific protein patterns

The demonstration in the present study of a female specific protein (FSP) in the serum of lobsters reflects vitellogenic activity, and adds support to the notion that synthesis of female specific proteins may be a general feature of crustacean reproductive biology. Croisille et al, in a recent review (1974) have discussed other species of crustaceans in which such a protein has been identified. The existence of female specific proteins in many insect species (Telfer 1954; Thomas and Nation 1966; Engelmann and Penney 1966; Engelmann 1969; Pan and Wyatt 1971) as well as crustaceans indicates similarities in vitellogenesis

among arthropods. The presence of sex-linked blood proteins in the toad, Xenopus laevis (Wallace and Dumont 1968; Wallace 1970; Redshaw and Follett 1971; Wallace and Bergink 1974) and the chicken, Gallus domesticus (Heald and McLaughlin 1965) indicates that vitellogenic proteins are a general characteristic of those vertebrates and invertebrates that produce a 'yolky' egg.

4. The crustacean female specific protein

Crustacean female specific proteins, immunologically identical with ovarian proteins, have been demonstrated in several species (Kerr 1966, 1969; Barlow and Ridgeway 1969; Horn and Kerr 1969; Wolin, Laufer and Albertini 1973; Croisille et al 1974; Fyffe and O'Connor 1974). In each instance, as is the case in the lobster, maximal concentrations of FSP in the serum of females precedes ovarian maturation and oviposition.

The occurrence of FSP in lobsters has an interesting analogy with the vitellogenic proteins in insects. Insect vitellogenic proteins are synthesized exclusively by the female fat body (Engelmann 1969; Pan et al 1969; Hagedorn and Judson 1972; Gelti-Douka et al 1974) coincident with the appearance of vitellogenins in the hemolymph. Several investigators have shown that proteins synthesized by the insect fat body are precipitated by antibodies to female specific proteins (Engelmann 1969; Pan et al 1969). Since these studies confirm an extra-ovarian site as the source of vitellogenic proteins, the hemolymph is presumed to transport the newly synthesized proteins to the oocytes in the ovary. Demonstration of micropinocytosis at the surface of the

insect oocyte (Anderson 1964; Roth and Porter 1964; Bell 1969; Bell and Barth 1970) suggests that vitellogenins are ingested as macromolecules, and incorporated into yolk globules in the ooplasm.

5. Possible sources of crustacean female specific protein

The highest levels of FSP in the lobster occur in the serum prior to maximum accumulation of yolk in the oocyte (Figures 10, 12,13,14) which strongly suggests that this protein is synthesized outside the ovary and is transported via the hemolymph to the oocytes. Work by others has postulated at least two possible sources of crustacean female specific protein: an extra-ovarian site, probably the hepatopancreas (Kerr 1968, 1969; Wolin et al 1973) and de novo synthesis by the oocyte itself (Beams and Kessel 1963; Kessel 1968; Ganion and Kessel 1972; Lui et al 1974). In an attempt to demonstrate extra-ovarian synthesis of vitellogenins, Kerr (1968) showed that hepatopancreas and hemocytes of the blue crab, Callinectes sapidus synthesized and secreted proteins in short-term culture, but was unable to show conclusively that the proteins were female specific. Wolin et al (1973) working with three species of decapod crustaceans (Uca pugilator, Libinia emarginata, and Cambarus clarkii) showed that a female specific protein was present in the serum of vitellogenic females prior to oviposition and that fluorescein-conjugated derivatives of the major yolk protein, lipovitellin (Wallace et al 1967), or Trypan blue, were taken up by oocytes most rapidly during the period when female specific protein concentration in the serum was maximal. They concluded that yolk

spheres in crustacean oocytes develop primarily from extra-ovarian lipovitellin micropinocytosed from the hemolymph. Other supporting evidence for this hypothesis is found in the observations of Hinsch and Cone (1969) and Zerbib (1973) that there are micropinocytotic pits at the surface of developing oocytes in the crab, Libinia emarginata and the amphipod, Orchestia gammarella respectively.

Evidence supporting the oocyte as the site of synthesis of yolk protein is ultrastructural (Beams and Kessel 1963; Kessel 1968; Ganion and Kessel 1972) and biochemical (Lui et al 1974). The ultrastructural evidence hinges on the presence in the developing oocyte of an extensive system of rough endoplasmic reticulum (Beams and Kessel 1963) in which the yolk granules are presumed to develop. Ganion and Kessel (1972) acknowledge the existence of pinocytotic profiles at the oocyte surface, but argue that any contributions of proteins from an extra-ovarian source are minor; however, they did not test uptake of the female specific protein. Their conclusions, therefore, are premature. Lui et al (1974) found that ovarian fragments take up and incorporate ^{3}H-leucine into lipovitellin in vitro. Since observations made in this study indicate that the major ovarian fraction (MOF) has at least 3 subunits (Figure 6c), it is possible that the female specific protein, immunologically identical to a major yolk protein (Figures 7,8) is taken up and chemical addition (e.g. the addition of leucine) occurs in the oocyte. This addition could account for the observations of Lui et al (1974).

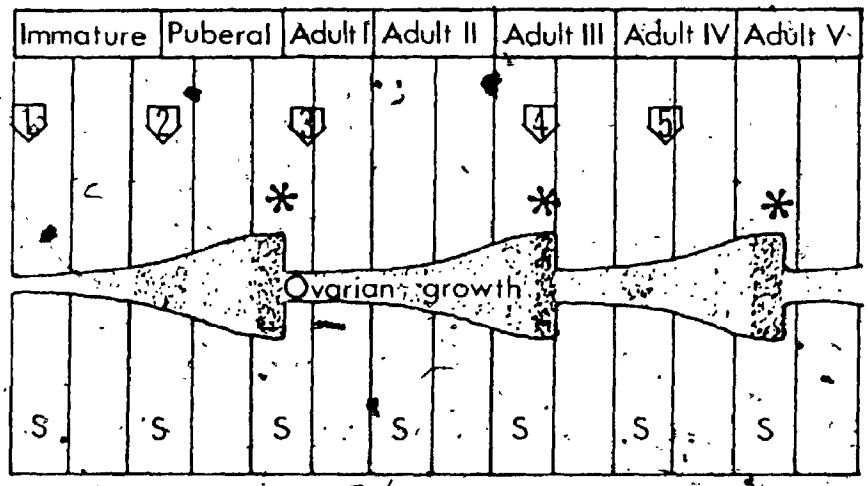
Proponents of either the ovarian or extra-ovarian site of synthesis of FSP have carefully avoided refutation of the opposite point of view. The ultrastructural evidence for intra-oocytic synthesis does not account for the circulating levels of FSP since export of the yolk proteins from the oocyte has not been shown. Stephens (1952) maintains that reabsorption of yolk from crayfish ovaries, which regularly occurs as a result of fluctuating temperature and photoperiod, accounts for the yolk proteins found in the hemolymph; but, in the lobster, reabsorption was observed to occur only occasionally. Evidence for an extra-ovarian site is weakened by the fact that the extra-ovarian site has not been adequately defined. Since both oocyte, and possibly hepatopancreas, are potential sources of vitellogenins in Crustacea, with some evidence for both, perhaps the best interpretation of the available evidence is that the total yolk present in the oocyte (including perhaps more than one type of protein) is the result of synthesis at both sites. Final resolution of this question, however, must await experiments designed to define the site of synthesis.

RELATIONSHIP BETWEEN MOLTING AND REPRODUCTION

Field studies on lobster reproduction (Squires 1970; Ennis 1971; Krouse 1973) have largely ignored the relationship between molting and oviposition, but do point out that the occurrence of molting and oviposition are seasonal; they both occur during May-October, when water temperatures are maximal (approximately 10-15°C). To define the relationship between molting and oviposition, lobsters were observed under laboratory conditions over two full seasons at ambient conditions.

The results show that the reproductive cycle of the female is characterized by seasonal vitellogenesis and oviposition alternating with the molting cycle (Figures 12,13,14). The observations on the normal female cycle are consistent with a hypothetical scheme for the relationship between molting and oviposition over several seasons (Aiken and Byard, unpublished, 1973; Figure 29). Immature females undergo annual molts, with the last two molts characterized by an increase in the abdominal width/carapace length ratio to approximately 64% or greater (Molts 1 and 2, Figure 29). Following a summer molt (Puberty II, Figure 29), oviposition takes place early the following summer. The lobster is now considered an Adult I. Molting in these individuals is delayed until early winter or the next spring (Adult II, Figure 29). The following summer and fall, molting, then oviposition occur within two months of each other (Adult II, Figure 29). After this, it is assumed that molting and oviposition continue to alternate, although as females become larger, they are increasingly difficult to find in the population (Ennis 1971). The hypothetical scheme is consistent with field

Figure 29: Hypothetical relationship between lobster molting (large arrows) and oviposition (*), over several seasons. Prior to, and including molt 1, lobsters are immature. Molt 2 represents the 'puberty' molt, and in the spring of the third season, the first oviposition occurs. Stippling indicates the approximate intensity of vitellogenesis. (See also Figures 22-24). Summer (S) includes June to October, and includes the period when water temperature is between 10-15°C.
(After Aiken and Byard, unpublished, 1973).


Immature
Puberal
Adult I
Adult II
Adult III
Adult IV
Adult V
1
2
3
4
5
Ovarian growth
S
S
S
S
S
S
S

29

observations in that spring and fall spawners have been reported (Squires 1970; Ennis 1971) which probably correspond to Adult I lobsters (spring) and Adult III lobsters (fall). Whether molt or oviposition occurs in the spring or fall seems to depend on the size group within the population; the larger animals molt and oviposit later in the season than do the small animals (Figures 1,2,3,4,29). In either case though, both events usually occur between June and October (Figures 1,2,3,4) which indicates that temperature plays an important role in stimulating these events. Photoperiod apparently plays a minor role in lobster growth at temperatures above 10°C (Aiken, personal communication).

Lobsters in the size range observed in this study (less than 90 mm carapace length) alternate molt and oviposition. That is not to say that one process is dependent on the other. If this were so, one would expect a better correlation between molt and oviposition. In mature female lobsters, either molt, oviposition, or both are seen to occur in any one season (Figures 1,2,3,4,29; Table V) indicating a degree of independence for each process. It is apparent that a temporal separation of ecdysis and oviposition is necessary since it is occasionally observed (Aiken, personal communication) that lobsters with ovaries in stage V that are also in late premolt (stages D_1-D_3) will not oviposit, but will instead reabsorb the yolk from the oocytes. This temporal separation seems to have a selective advantage for the lobster, since molting immediately after oviposition would result in the loss of the newly laid egg mass. Synthesis of new cuticle and vitellogenic

proteins place major demands on metabolic reserves, thus it appears essential that the two processes must be temporally separated.

ENDOCRINE CONTROL OF VITELLOGENESIS

1. General considerations

Since Panouse (1943, 1944) first showed that a substance contained in the eyestalk X-organ-sinus gland complex of Palaemon serratus inhibited vitellogenesis, ablation and implantation experiments have shown that this inhibiting factor exists in at least 15 species of the suborders Natantia and Reptantia (Table 1 of Adiyodi and Adiyodi 1970). The general conclusion reached from these studies is that the eyestalk factor, called ovarian inhibiting hormone (OIH), is present at high levels in prepuberal and mature females during periods of ovarian quiescence, and at some point, in response to either temperature or photoperiod, the levels are reduced allowing vitellogenesis to proceed. Eyestalk ablation prior to vitellogenesis removes the inhibition, resulting in precocious vitellogenesis. Once vitellogenesis is underway, (i.e. OIH presumably already low) eyestalk ablation has no effect. This effect of ablation is seen during the normal breeding season (Brown and Jones 1949; Aoto and Nishida 1956; Passano and Jyssum 1963; Weitzman 1964; Demeusy 1965; Gomez 1965; R.G. Adiyodi 1968a) as well as other periods (Panouse 1943,1947; Otsu and Hanoaka 1954; Carlisle 1953; Demeusy 1965).

The results obtained by removal of the ovarian inhibiting hormone could be interpreted as either a direct effect on the ovary or an indirect effect via an intermediate gland, which, in turn, stimulates vitellogenesis. The mechanism may be similar to the effect of juvenile hormone in stimulating the synthesis of

vitellogenic proteins in the fat body of the insect (Engelmann and Penney 1966; Engelmann 1969). In addition to direct induction of yolk protein synthesis by the fat body, juvenile hormone has also been shown to facilitate uptake of yolk proteins by the oocytes (Luscher 1968; Pratt and Davey 1972). The crustacean mandibular organs (LeRoux 1968,1969,1974) which are separate from the Y-organs (Sochasky et al 1972; Aoto et al 1974) have been proposed as an analog of the corpus allatum (Adiyodi and Adiyodi 1970). This idea is particularly attractive since a female specific protein has been demonstrated in several crustaceans. The existence of a corpus allatum analog would confirm Passano's postulate (1960) of a general arthropod mechanism for vitellogenesis.

2. Mandibular organ structure

The lobster mandibular organ has the general organization of an endocrine structure (cf the description of vertebrate endocrine structures, Bloom and Fawcett (1968)). Extensive vascularization provides the parenchymal cells with immediate access to the hemolymph (Figures 16,17) and there are no ducts for the export of its secretory products. At the ultrastructural level, the most characteristic feature of the mandibular organ cells is the quantity of two distinct types of agranular endoplasmic reticulum: tubular endoplasmic reticulum (TER; Figures 18,21) and cisternal endoplasmic reticulum (CER; Figures 21,22). Occasionally the agranular reticulum is so extensive that it displaces other cytoplasmic organelles (Figures 21,22).

A comparison of the characteristics reported here for Homarus americanus mandibular organ with those for Palaemon paucidens (Aoto et al 1974) reveals similarities between the glands at the light microscopic level, but marked differences at the ultrastructural level. The mandibular organ in Palaemon has little agranular endoplasmic reticulum and no microbodies. In Homarus mandibular organs however, the endoplasmic reticulum is abundant, proliferates in mid-to-late premolt (Figure 21), and is associated with a large population of microbodies (Figures 18,22,23). Electron microscopic observations of the mandibular organs in the crab Carcinus maenas and the crayfish, Orconectes virilis show that these glands differ from that of the lobster in the extent of the microbody population (Byard, unpublished observations).

Agranular TER is a common feature of vertebrate cells involved in steroid synthesis (Long and Jones 1967; Christensen and Gillim 1969; Lofts 1972). On the basis of a well developed agranular TER, Locke (1969) has suggested that insect oenocytes are involved in steroid metabolism. Agranular TER is prominent in some insect corpora allata (Fukuda et al 1966; Odhiambo 1966a,b; Aggarwal and King 1969; King et al 1969; Thomsen and Thomsen 1970), and has been implicated as the site of synthesis of juvenile hormone (Röller and Dahm 1968). 'Formed' secretory product has not been found in steroid synthesizing cells, or in the cells of the corpora allata, and this observation is consistent with the idea that the extensive cisterns of TER contain the secretion product in a non-condensed form. Thus, the mandibular organ cells, with their

well developed agranular TER are morphologically similar to cells involved in either lipid or steroid synthesis, and may be responsible for synthesis of a similar compound in the lobster. Further indication that the mandibular organ cells are involved in lipid synthesis is a large population of microbodies and lipid droplets, both characteristic of vertebrate cells involved in lipid synthesis (Beard 1972). It is clear that on morphological grounds that the mandibular organs are similar to the corpus allatum.

3. The role of the mandibular organ

Since the mandibular organ was described only recently, (LeRoux 1968,1969,1974; Aoto et al 1974; Byard et al 1975) there are no studies describing its function in any crustacean. The present study attempts to assign a role to this structure, particularly in relation to the synthesis of hemolymph proteins. Bilateral ablation of the mandibular organs was shown to have: (1) no effect on the time of molting (in the time taken to the first postoperative molt, Table VI); (2) no short-term effect (three months) on total serum protein concentrations or the level of female specific protein prior to normal oviposition (Figures 25, 26); and, (3) a long-term effect (18-24 months) of reducing total serum protein concentration (Figure 28) but without an effect on female specific protein concentration (Figure 27). The absence of a mandibular organ mediated effect on molting confirms the observations made by Sochasky et al (1972), Aoto et al (1974), and LeRoux (1974) that this organ differs from the molting gland of Echalier (1959), and contains no molt-inducing substances (Carlisle and Connick 1973a,b).

The long-term effect of reduced total serum protein concentration in lobsters deprived of their mandibular organs (Figure 28) is similar to that observed in some insects after removal of their corpora allata. It has been shown in many insects that allatectomy reduces the rate of protein synthesis by the fat body (reviewed by Steele 1975), including the synthesis of vitellogenic proteins. In certain insects, allatectomy also results in an accumulation of vitellogenins in the hemolymph, probably as a result of impaired uptake of these proteins by the oocytes (Highnam et al 1963; deLoof and deWilde 1970). This latter observation suggests that vitellogenins have a slow rate of degradation. Similarly, Wallace and Dumont (1968) showed that vitellogenic proteins in the toad, Xenopus laevis had a slow rate of turnover and degradation. After removal of the mandibular organ in the lobster, the female specific protein is maintained at normal concentrations in the serum in spite of declining total serum protein (Figures 27,28) indicating a probable depression in non-specific protein synthesis in the hepatopancreas. It may be that female specific protein synthesis is also depressed but its turnover and degradation is slower than that of the other serum proteins. The long period required for the disappearante of reabsorbed yolk (Figure 15) from the serum supports this idea. Final resolution of this question must await definitive experiments on both the site of synthesis and the rate of turnover of the female specific protein.

Short-term ablation experiments indicate that the mandibular organs are not required for final vitellogenesis and oviposition. Serum proteins are unaffected in the short term (Figures 25,26) suggesting that the effect of mandibular organ ablation may be stage specific. A comparable situation exists in allatectomized insects where hemolymph protein levels are unchanged for several days or weeks after ablation (Steele 1975). The absence of the mandibular organ, however, does cause a decrease in total serum protein, but not the female specific protein over a long period of time. It may be that the mandibular organ is only required well in advance of reproduction, thereby providing a trigger for subsequent events, including synthesis of the appropriate proteins, although not the female specific protein.

4. Ovarian control of vitellogenesis

Evidence for an alternative control mechanism for the production of female specific protein in crustaceans is derived from the experiments of Croisille et al (1974) and Meusy et al (1971) on the amphipod, Orchestia gammarella. In this species secondary sexual characters are determined by the presence or absence of androgenic glands, structures associated with the male reproductive tract (Charniaux-Cotton 1954, 1965). Implantation of androgenic glands into immature or mature females brings about masculinization of the host, including an inhibition of ensuing vitellogenesis with a concomitant decline and ultimate disappearance of the female specific protein. In the reverse experiment (removal of the androgenic glands from a male), feminization follows;

that is, spermatogenesis is arrested, and oocytes appear in the gonad, but female specific protein does not appear. Implantation of an ovary (even an immature one) into these operated males results in the appearance of female specific protein, and the graft undergoes vitellogenesis; therefore, either the implanted ovarian tissue is stimulating synthesis of the female specific protein at an extra-ovarian site, or the implanted ovary is the site of synthesis of this component. In either case, the experiment demonstrates that synthesis of female specific protein cannot occur in the absence of the ovary, and, in fact, may be controlled by it. Ovarian control of the synthesis of female specific proteins has also been shown in the toad, Xenopus laevis (Wallace and Dumont 1968; Wallace and Bergink 1974) and the mosquito Aedes aegypti (Hagedorn and Fallon 1973; Hagedorn 1974). The persistence of normal levels of female specific protein in the serum of lobsters deprived of their mandibular organs while the concentration of other proteins is declining, strongly suggests that the female specific protein is not under the control of the mandibular organ, but may be, as suggested by Croisille et al (1974), a secondary sexual characteristic under the control of the ovary.

CONCLUSION

Although the concentration of lobster female specific protein during the reproductive cycle, and its relationship to the molting cycle has been detailed by this study, the control of vitellogenesis remains enigmatic. The mandibular organ does not appear to have a major role in modifying FSP levels, and therefore, seems to yield a poor analogy with the insect corpus allatum. Additional evidence, from the work of others (reviewed by Adiyodi and Adiyodi 1970; Croisille et al 1974), for the control of vitellogenesis and FSP suggests a role for both the eyestalk ovarian inhibiting hormone (OIH) and the ovary itself. It is conceivable that removal of OIH results in the release of an ovarian hormone which stimulates synthesis of vitellogenic proteins at an unknown site, probably the hepatopancreas. In order to design meaningful experiments to determine control mechanisms for vitellogenesis in crustaceans, the basic experiments to establish the site of synthesis of the yolk proteins are required. Until this site is known, the present literature on the mechanisms for hormonal control can only remain speculative.

SUMMARY

1. Five stages (I-V) of lobster ovarian development were recognized by examining both color and relative weight of the ovary. Stages II-IV represent the period when the ovary is enlarging due to the accumulation of yolk in the oocytes.
2. Both molt and oviposition were found to occur between June and November. Molt, oviposition, or both were observed within in any one season, depending on the size of the lobster within the population. Reabsorption of yolk proteins from the ovaries was occasionally seen to occur. The asynchronous timing of molting and oviposition suggests that these processes are somewhat independent, although interrelated.
3. A female specific protein (FSP), immunologically identical to a major protein extractable from the mature ovary (MOF), was found in the serum of vitellogenic females. FSP is absent from the serum of males and immature females, and probably represents a yolk protein in transit to the ovary.
4. Serum FSP is detectable in mature, non-ovigerous, intermolt females with ovaries in stages II-IV, with a maximum level (12.6 mg/ml hemolymph) occurring when the ovary is in stage III (O_f approximately 175). In late premolt (stages D_1 or later) to postmolt (stage B), ovaries were found in stages I-IV, but levels of serum FSP are low, suggesting that premolt events inhibit vitellogenesis.

5. Vitellogenesis, as reflected by serum FSP concentrations, occurs largely during periods when water temperatures are greater than 10°C (June to October) and is most intense in the 2-4 month period prior to oviposition.
6. The mandibular organ (MO) has the general organization of an endocrine organ, and its cells are ultrastructurally similar to cells that synthesize steroids or lipids.
7. Bilateral MO ablation results in: (a) low total hemolymph protein levels over the long term, but no effect on total hemolymph protein levels over the short term; (b) no short or long term effect on FSP levels; (c) no effect on either the occurrence of oviposition, or the duration of the intermolt period.
8. Although the lobster mandibular organ structurally resembles the insect corpus allatum, experimental evidence does not support an analogy between the two structures.

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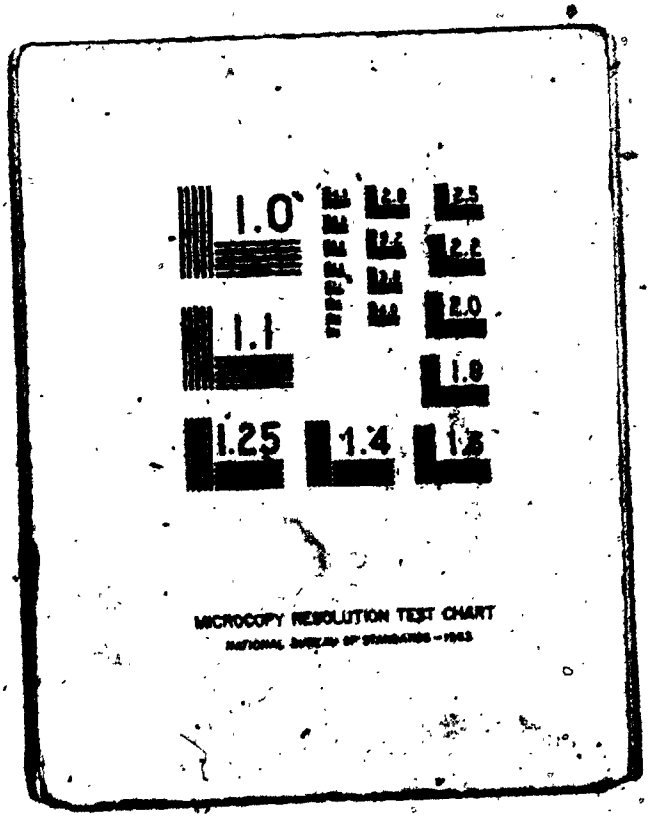

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Appendix I

Criteria for staging proecdysis from pleopods of Homarus. *

Molt stage	Pleopod stage	Description
C_4	0	Epidermis closely applied to cuticular nodes at tip of pleopod; no amber zone or epidermal retraction at pleopod tip.
D_o	1.0	First indication of apolysis - amber or double-bordered region forms at the pleopod tip. Chromatophores often show signs or reorganization but there is no epidermal retraction from the cuticle.
D_o	1.5	Epidermis retracting from terminal cuticular nodes; may have double-bordered appearance.
D_o	2.0	Epidermal line clearly formed and retracting from lateral cuticular nodes.
D_o	2.5	Maximum epidermal retraction - not touching any lateral cuticular nodes.
D_1'	3.0	Invagination papillae form at site of future setae; epidermis becomes scalloped.
D_1''	3.5	Invagination papillae clearly formed but shafts of new setae not well defined.
D_1'''	4.0	Shafts of developing setae visible but proximal ends not clearly defined. Shafts now invaginated to maximum length.
D_2'	4.5	Shafts visible full length but proximal ends are bifurcate instead of blunt. Barbules becoming visible on setal shafts.
D_2''	5.0	Shafts of developing setae thick, proximal ends blunt.
D_3'	5.5	Shafts of setae very thick and dark, proximal ends blunt. Classify as D_3'' if folds or ripples are visible in cuticle on upper surface of pleopod.

* Table 3 (page 1342) of Aiken (1973).

Appendix II

Stock solutions for polyacrylamide disc gel electrophoresis.

(Modified from Davis 1964). Solutions were usually remade after two months on the shelf except as noted. (Chemicals from Canalco Inc., Rockville, Maryland).

A.	1N HCl	24 ml (approx.)
	Tris	18.1 g
	Temed	0.12 ml
	Water	to 100 ml (pH 8.8)
B.	In HCl	48 ml (approx.)
	Tris	5.98 g
	Temed	0.46 ml
	Water	to 100 ml (pH 6.6-6.8)
C.	Acrylamide	28.0 g
	Bis	0.735 g
	Water	to 100 ml
D.	Acrylamide	20.0 g
	Bis	5.0 g
	Water	to 100 ml
E.	Riboflavin	4.0 mg
	Water	to 100 ml
*F.	Sucrose	40 g
	Water	to 100 ml
*G.	Ammonium persulphate	0.14 g
	Water	to 100 ml
H.	Tris-glycine	34.8 g
	Water	to 1000 ml (pH 8.8)

(* freshly prepared every three days

Running gel (small pore gel)

Solution A	1 part	
" C	1 "	
" G	2 "	(catalyst)

Final concentration of acrylamide = 7%

Stacking gel (large pore gels)

Solution B	1 part
" D	1 "
" E	1 "
" F	1 "
Water	1 part

Reservoir buffer

Solution H	1 part
Water	4 parts

Tracking dye

Bromophenol blue .005%